Early Posthatch Nutritional Strategies to Reduce the Incidence and Severity of Wooden Breast Myopathy

Dissertation

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Abstract

Wooden Breast (WB) myopathy is present within the broiler industry worldwide. The WB affected muscles are palpably hard under severe oxidative stress and inflammation. Posthatch muscle growth is dependent on satellite cells and are sensitive to nutritional changes early posthatch. Thus, satellite cells are able to be modified by nutritional strategies early posthatch and thereby alter the muscle structure. The overall objective of this study was to reduce the incidence of WB myopathy through early posthatch nutritional interventions including vitamin E (VE) and alpha lipoic acid (ALA) with antioxidant properties, and omega-3 (n-3) fatty acids with anti-inflammatory effect.

The first three aims determined the effects of VE (200 IU/kg) and n-3 fatty acids (n-6/n-3 ratio of 3.2:1) independently or in combination when fed during the starter phase (0 to 10 day) or grower phase (11 to 24 day) on WB severity, growth performance, meat quality, morphological structure of the pectoralis major (p. major) muscle and small intestine, and expression of genes likely associated with WB in p. major muscle and small intestine. It was found that VE supplementation during the starter phase or grower phase reduced the severity of WB myopathy both by palpation and by microscopic without sacrificing growth performance and meat yield in broilers at market age (58 days of age). In contrast, n-3 fatty acids supplementation in starter diets decreased final body weight and meat yield. Genes associated with muscle development and glucose metabolism were differentially expressed in the p. major muscle of the broilers supplemented with VE in the grower diet, indicating reduced muscle degeneration and lipid deposition. Genes involved

in gut nutrient transport, oxidative stress, and inflammation were differentially expressed in small intestine of the broilers supplemented with VE during the grower phase, indicating improved nutrient transport and reduced oxidative stress and inflammation. These suggest that VE supplementation especially during the grower phase may reduce the incidence of WB through improving muscle and intestinal morphology in broilers at market age.

The beneficial dietary effects on reducing WB severity was initiated from an early age. The objectives of the fourth and fifth aims were to identify the effects of VE (160 mg/kg) and ALA (500 mg/kg) on the developmental onset, severity, and progression of WB based on p. major muscle and small intestinal morphology and expression of genes associated with WB in broilers during the first 3 weeks posthatch. There was no phenotypic detection of WB by 3 weeks of age. However, microscopic changes associated with WB was detected beginning at 1 week of age in all groups. Supplementation of VE and ALA independently and in combination reduced microscopic WB severity at 2 and 3 weeks of age compared to the control. Expression of genes associated with adipogenesis and inflammation was reduced in VE, ALA, and combination of VE and ALA groups compared to the control at 3 weeks of age. These data suggest that VE and ALA supplemented independently and in combination had positive effects on mitigating WB severity, improving muscle and intestinal structures as early as 2 weeks of age, with combination of VE and ALA showing the most effective effect. The results from this study can be potentially transferred to the broiler industry reducing the serious economic loss that WB causes.

Dedicated To My Family

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Publications

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Field of Study

Major Field: Animal Sciences

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Chapter 1: Literature Review

1.1 Introduction

In the last few decades, the poultry industry has selected broilers with higher growth rate and breast muscle yield to meet the increased demand of consumers (Brewer et al., 2012; Chatterjee et al., 2019). As a result of these selection practices, myopathies have arisen in the breast muscle of modern commercial broilers (Dransfield and Sosnicki, 1999; Petracci et al., 2013; Tijare et al., 2016). Among the myopathies, Wooden Breast (WB) is of great concern to the poultry industry as WB has been discovered worldwide (Sihvo et al., 2014; Tasoniero et al., 2016; Xing et al., 2019) resulting in serious economic loss due to reduced meat quality and product downgrades (Kuttappan et al., 2016). Wooden Breast affected muscles are palpably hard (Sihvo et al., 2014), under severe oxidative stress (Mutryn et al., 2015; Abasht et al., 2016; Brothers et al., 2019), and inflammation (Mutryn et al., 2015; Zambonelli et al., 2017).

Posthatch muscle growth is dependent on myogenic stem cells, satellite cells, which are most active the first week posthatch (Halevy et al., 2000) and are sensitive to nutritional changes (Halevy et al., 2000; Velleman et al., 2010; Powell et al., 2014). When nutritional strategies are applied during the early posthatch period, long-term effects on muscle growth and meat quality will be produced (Noy and Sklan, 1999; Dangott et al., 2000). In addition, the early posthatch period is also essential for intestinal development (Noy et al., 2001; Uni et al., 2003a). Broiler chicks are sensitive to nutrition during this period in that sufficient early nutrient supply improves intestinal morphology and enhances intestinal development

(Noy et al., 2001; Batal and Parsons, 2002; Ao et al., 2012; Jha et al., 2019). With good intestinal development, broilers will have improved nutrient absorptive functions and gut health promoting animal growth including muscle growth (Jha et al., 2019) and potentially decrease the development of the myopathies. Therefore, nutritional interventions targeting reducing oxidative stress such as vitamin E (VE) and alpha lipoic acid (ALA), and reducing inflammation such as omega-3 (n-3) fatty acids during the early posthatch period will potentially improve breast muscle and intestinal growth and reduce the incidence and severity of WB myopathy.

This literature review begins with an overview of skeletal muscle structure and function, skeletal muscle growth and development, and how extracellular matrix regulates muscle growth and influenced by WB. Intestinal structure and function, and intestinal growth and development will be introduced next. These topics are then followed with meat myopathies including broiler genetic selection, novel broiler myopathies, and WB plausible etiologies. The last topic will be focused onpotential early posthatch nutritional interventions including VE, n-3 fatty acids, and ALA.

1.2 Skeletal muscle

Studies examining WB need to understand the skeletal muscle structure and function, skeletal muscle growth and development, and how extracellular matrix regulates muscle growth and is influenced by WB. Skeletal muscle, smooth muscle, and cardiac muscle make up the muscular system. Skeletal muscle accounts for 35%-50% of the body

weight (Webster, 1986; Topel and Kauffman, 1988). The skeletal muscle is attached to the bone jointed by collagen fiber bundles and is striated muscle under voluntary nerve control (Kuno, 1915; Spiro, 1956). The skeletal muscle supports the functions of postural support, locomotion and homeostasis (Terjung and Hood, 1986; Henriksson, 1992; Hocquette et al., 1998).

1.2.1 Skeletal muscle structure and function

Skeletal muscle is composed of muscle fiber bundles surrounded by a connective tissue framework (Figure 1.1; Strohman et al., 1990). Outside the entire muscle is a connective tissue layer called the epimysium. The muscle fiber bundles are separated from each other by the connective tissue layer called the perimysium. Another connective tissue layer called the endomysium is formed surrounding each muscle fiber inside the muscle fiber bundle (Rowe, 1981). The connective tissue layers are joined together to provide structural support for the muscle (Parry and Craig, 1984).

Muscle fibers are made up of myofibrils containing sarcomeres, which are responsible for the muscle contractile ability (Wang and Ramirez-Mitchell, 1983; Morgan, 1985). One sarcomere is defined as the distance between two adjacent Z lines (Franzini-armstrong, 1973). Sarcomeres are composed of thick and thin filaments (Ken'ichi and Hiroshi, 1979). The thick filaments containing primarily myosin and titin, are anchored at the M line (Gilev, 1962). The thin filaments including mainly actin, tropomyosin, troponin, tropomodulin, and nebulin, are anchored at the Z line (Spiro, 1956). The myosin filaments overlap with the actin filaments in the A zone but not overlap in the H zone (de Villafranca

and Marschhaus, 1963). The plasma membrane of the muscle cell, sarcolemma, contains invaginations into the muscle fibers at regular intervals called the transverse tubule system (Ishikawa, 1965). When an action potential arrives, it spreads inside the muscle fiber through T-tubules (Strickholm, 1966) and causes the sarcoplasmic reticulum, an internal membrane system within the muscle fiber, releasing calcium ions (Huxley, 1974). The calcium ions bind to troponin resulting in a conformational change between tropomyosin and actin, which exposes the myosin binding site and allows the myosin binding to actin forming a cross-bridge between myosin and actin (Gilev, 1962; Bennett et al., 1999). The adenosine triphosphate is hydrolyzed to adenosine diphosphate converting the stored chemical energy to the mechanical force of muscle contraction (Fukazawa et al., 1963; Takahashi et al., 1965).

There are three types of skeletal muscle fibers (Table 1.1; Aberle et al., 1979). The red slow twitch oxidative (type I) fibers generate energy through oxidative metabolism. This type of fiber has a higher aerobic capacity with a higher capillary density (Sams and Janky, 1990). The fast-twitch, oxidative (type IIA) fibers are intermediate fibers. The white fast twitch glycolytic (type IIB) fibers metabolize glycogen and glucose to lactate with lower blood supply (Aberle et al., 1979). The chicken breast muscle is composed of type IIB fibers having fewer capillaries (Smith and Fletcher, 1988).

As type IIB fibers are glycolytic fibers metabolizing glycogen and glucose, lactate is a by-product of anaerobic metabolism. With lower blood supply in type IIB fibers, lactate is more prone to not be removed and remained in muscle resulting in lower pH and muscle

damage (Velleman et al., 2003; Mutryn et al., 2015). Additionally, oxygen supply can be restricted through fewer capillaries. When oxygen is not reduced properly, reactive oxygen species (ROS) with oxidizing ability can be produced contributing to oxidative stress (Voljč et al., 2011; Panda and Cherian, 2014), which is closely associated with WB myopathies (Mutryn et al., 2015; Abasht et al., 2016; Brothers et al., 2019). Therefore, type IIB muscle fibers like the breast muscle are more prone to be affected with WB myopathy compared to the other types of fibers.

1.2.2 Muscle growth and development

There are two phases during skeletal muscle growth including the embryonic phase of fiber formation and posthatch phase of muscle growth. In embryonic phase, gastrulation results in reorganization of the embryo from spherical blastula into multi-layered structure, containing endoderm, ectoderm, and mesoderm (Schoenwolf et al., 1992; Schoenwolf and Smith, 2000). Mesodermal cells proliferate and separate into somites (Winklbauer, 1990; Christ and Ordahl, 1995). Embryonic myoblasts migrate along the somites and begin to proliferate (Schoenwolf et al., 1992; de Angelis et al., 1999). As shown in Figure 1.2, mononucleated myoblasts fuse with adjacent ones to form primary muscle fibers (Moss and Leblond, 1971). Stepwise formation leads to the formation of the secondary fibers. At the time of hatch, the myoblasts are withdrawn from the cell cycle and myofiber formation is complete (Smith, 1963). The embryonic formation of muscle fibers is referred to as hyperplasia (Antonio and Gonyea, 1993).

By the time of hatch, posthatch muscle growth is dependent on myogenic stem cells called the satellite cells (Mauro, 1961). Satellite cells fuse with multinucleated myofibers, donate their nuclei, and increase protein synthesis capabilities (Moss and Leblond, 1971). This results in muscle growth through hypertrophy or the enlargement of existing muscle fibers (McCormick and Schultz, 1992). Muscle growth after hatch is most active the first week posthatch and then gradually become quiescent (Halevy et al., 2000). However, satellite cells can be reactivated to undergo regeneration process repairing the damaged fibers or forming new muscle fibers when the muscle is damaged (Figure 1.3). The self-renewal of the satellite cells is required for generation of new muscle fibers to maintain satellite cell pool (Collins et al., 2005). The satellite cells attach to the existing muscle fibers and proliferate, fuse with the damaged muscle fibers or form new muscle fibers (Bischoff, 1975; Schultz, 1989). Sufficient blood supply is required for regeneration (Christov et al., 2007; Rhoads et al., 2009). The breast muscle composed of type IIB muscle fibers have lower vascularization (Smith and Fletcher, 1988). Moreover, excessive giant muscle fibers from selection for higher breast muscle yield restricts the area for connective tissue and capillaries in heavy weight fast growing broilers (Dransfield and Sosnicki, 1999; Velleman et al., 2003). With reduced blood supply, regeneration of the damaged muscle fibers mediated by satellite cells are reduced. If satellite cells cannot repair the damaged muscle fibers properly, extensive connective tissue will replace the muscle fibers contributing to fibrosis, which is one of the major characteristics of WB (Sihvo et al., 2014; Velleman and Clark, 2015). The regenerated muscle should be similar to the original one

morphologically and functionally. However, Velleman et al. (2018a) found that muscle fibers in WB affected tissues were not fully regenerated with more small-diameter myofibers and reduced sarcomere organization.

Muscle growth and development during both embryonic and posthatch phases requires the expression of myogenic regulatory factors (Figure 1.2). Myogenic determination factor 1 (MyoD) and myogenic factor 5 (Myf5) are necessary for myoblast formation and satellite cell proliferation (Rudnicki et al., 1993). Myogenin is needed for multinucleated myotube formation (Hasty et al., 1993). Muscle regulatory factor 4 (MRF4) is required for the myofiber formation (Hinterberger et al., 1991).

Satellite cells are sensitive to nutritional changes (Halevy et al., 2000; Velleman et al., 2010; Powell et al., 2014) and will have long-term effects on muscle growth and meat quality (Noy and Sklan, 1999; Dangott et al., 2000; Velleman et al., 2014). Halevy et al. (2000) evaluated the effect of feed deprivation on breast muscle growth in chicks suggesting that sufficient nutrients during the first week posthatch are critical for maximal muscle growth. Velleman et al. (2010) reported that feed restriction during the first week posthatch altered expression of genes required for proliferation and differentiation of muscle cells and reduced p. major muscle weight. Increased myofiber necrosis and fat deposition in broilers have also been found when feed restriction was applied during the immediate posthatch (Velleman et al., 2014). Additionally, satellite cells are stem cells that can transdifferentiate into other cell types such as adipocytes when they are under appropriate stimuli (Powell et al., 2014; Velleman et al., 2014). In this way, satellite cells

are able to be modified by early posthatch nutritional strategies and thereby influence muscle structure and meat quality.

1.2.3 Extracellular matrix

The extracellular matrix (ECM) plays an important role in muscle growth regulation (Melo et al., 1996; Velleman, 1999). It is an organized dynamic network of secreted molecules that provides structural substance for the surrounding cells (Aumailley and Gayraud, 1998) and regulates cellular activities associated with cell migration (Burridge and Fath, 1989; Choquet et al., 1997), growth factor activities (Ignotz and Massague, 1986; Nakagawa et al., 1989; Hildebrand et al., 1994; Taipale and Keski-Oja, 1997), and signal transmission modulating proliferation and differentiation (Lin and Bissell, 1993; Streuli, 1999). The composition of the ECM changes according to the cellular needs (Streuli, 1999). The major components of the ECM include collagen, proteoglycan, and non-collagenous glycoproteins.

Collagen is the most abundant protein of the ECM in muscle. At least 29 different types of collagen have been found (Söderhäll et al., 2007). Collagen is a right handed triple helix (Brodsky and Ramshaw, 1997) consisting of the amino acid Gly-X-Y repeat, in which X and Y can be any amino acids but usually are proline and lysine (Nemethy and Scheraga, 1982). Among them, type IV collagen is the primary structural constituent of the basement membrane (Trueb et al., 1982; Brazel et al., 1987). It forms a network interacting with other extracellular matrix molecules like proteoglycans and non-collagenous glycoproteins (Vercellotti et al., 1985; Aumailley and Gayraud, 1998). Through these complex

interactions, type IV collagen is involved in various cellular activities such as cell adhesion and cell migration (Herbst et al., 1988; Chelberg et al., 1989). Type IX collagen is mostly found in cartilage and eye virtous, which is responsible for collagen fibrils stabilization and collagen fibril diameter control (Muller-Glauser et al., 1986; Savontaus et al., 1998). Lack of type IX collagen is related with the early onset of osteoarthritis (Nakata et al., 1993).

Type I and III are the predominant fibrillar collagens in skeletal muscle (Duance et al., 1977; Sasse et al., 1981; Mayne and Sanderson, 1985). They play key roles in structural support and tensile stress transmission providing mechanical stability to the muscle (Ohtani et al., 1988; Kannus, 2000). The fibrillar collagen aligns in a quarter-stagger array permitting crosslink formation. The formation of crosslinks between individual collagen is necessary for collagen fiber formation (McCormick, 1994). The crosslinks are covalent bonds either divalent or trivalent and determine the stabilization and function of the collagen (Feit et al., 1989). The divalent ketoamine collagen crosslink forms first which is reversible and can be replaced further by a nonreversible trivalent hydroxylysylpyridinium (HP) crosslink (McCormick, 1994). The HP crosslink increases with age (Palokangas et al., 1992) with higher amounts of the HP crosslink resulting in less tender meat (McCormick, 1999).

The palpable hardness in WB affected breast muscle is associated with the high levels of highly crosslinked fibrillar collagen (Velleman and Clark, 2015; Velleman et al., 2017; Tonniges et al. 2019). Velleman et al. (2017) compared the collagen fibrils in three

different genetic broiler lines. In a fast growing line with high frequency of WB phenotypic detection, tightly packed collagen fibrils were identified. In another fast-growing line with low frequency of WB phenotypic detection, collagen fibrils were randomly aligned. In the slow growing line with no WB detected, collagen was sparsely distributed. The tightly packed collagen is associated with a high level of crosslinking resulting in the hardness of p. major muscle. In contrast, the diffused collagen with a low level of crosslink does not impact the toughness of the breast muscle.

Proteoglycan is another major component of the ECM. It is composed of a core protein covalently attached with one or more glycosaminoglycan (GAG) chains (Hardingham and Fosang, 1992). Glycosaminoglycans can be categorized into four types including heparan sulfate, chondroitin sulfate, dermatan sulfate, and keratan sulfate. Proteoglycans are related with various biological activities such as regulation of growth factors (Yumaguchi et al., 1990; Flaumenhaft and Rifkin, 1991; Schlessinger et al., 1995; Taipale and Keski-Oja, 1997), cell growth (Wight et al., 1992; Iozzo, 1997), water-holding capacity (Beaty and Mello, 1987; Yanagishita, 1997), and organization of the extracellular environment (Woods et al., 1984; Bernfield and Sanderson, 1990; Yanagishita, 1997).

Aggregating proteoglycans are large proteoglycans having molecular weights of over 1 million daltons. They are made up of over 100 GAG chains contributing to a high negative charge, which allows them ionically interact with water and be associated with tissue hydration (Heinegard and Sommarin, 1987). Cells will become closely packed with deficient aggregating proteoglycans leading to numerous diseases such as osteoarthritis

(Malemud, 1991; Rizkalla et al., 1992) and the cartilage defect nanomelia (Velleman and Clark, 1992; Primorac et al., 1994). In terms of non-aggregating proteoglycans, decorin is a small arch-shaped leucine-rich proteoglycan (Weber et al., 1996; Hocking et al., 1998). It is composed of a central core protein and a single covalently attached chondroitin or dermatan sulfate chain (Weber et al., 1996). Collagen crosslinks are regulated by decorin (Keene et al., 2000; Velleman and Clark, 2015). The decorin binds to the fibrillar collagen triple helix to mediate collagen crosslink formation (Weber et al., 1996). In addition, decorin can regulate muscle growth by interacting with growth factors such as transforming growth factor-β (TGF-β) (Hildebrand et al., 1994) and myostatin (Miura et al., 2006). The TGF-β and myostatin are known to be strong inhibitors of satellite cell proliferation and differentiation. When the core protein of decorin binds to these two growth factors, both TGF-β (Droguett et al., 2006) and myostatin (Miura et al., 2006) can be sequestered from their receptors and therefore stimulate proliferation and differentiation.

Syndecan-4 is an important transmembrane heparan sulfate proteoglycan that is involved in cellular activities associated with cell proliferation and differentiation (Longley et al., 1999; Woods et al., 2000; Velleman et al., 2007, 2018b; Shin et al., 2012; Harthan et al., 2013). Syndecan-4 is composed of a core protein covalently attached with three heparan sulfate GAG chains and two N-glycosylated chains (Lee et al., 1998; Bernfield et al., 1999; Couchman et al., 2001). The cytoplasmic domain of the syndecan-4 can bind to protein kinase C alpha through the assistance of phosphatidylinositol 4,5-biphosphate (PIP2) (Lee et al., 1998; Horowitz et al., 1999; Song et al., 2012a) and further activates ras

homolog gene family member A (RhoA) (Dovas et al., 2006; Shin et al., 2013). The activation of RhoA affects focal adhesion formation and influences cell migration and proliferation (Woods et al., 2000; Dovas et al., 2006; Velleman et al., 2008; Song et al., 2012b).

Syndecan-4 has been shown to be associated with fibroblast growth factor 2 (FGF2) cell signaling (Volk et al., 1999; Velleman et al., 2007, 2008; Zhang et al., 2008; Song et al., 2011). Fibroblast growth factor 2 stimulates muscle cell proliferation and inhibits differentiation (Dollenmeier et al., 1981). Syndecan-4 regulates cellular responsiveness to FGF2 through binding FGF2 to its heparan sulfate chains and core protein with cytoplasmic domain interacting with PIP2 (Song et al., 2012a; Shin et al., 2013). Additionally, expression of the muscle regulatory factors (MRFs) is regulated by syndecan-4. With syndecan-4 gene knockdown, expression of MyoD and MRF4 was increased during cell proliferation (Shin et al., 2012; Harthan et al., 2013), which could impact muscle formation and muscle yield.

Syndecan-4 is also involved in integrin mediated fibronectin induced focal adhesion formation (Saoncella et al., 1999; Morgan et al., 2007). Integrin is a glycoprotein composed of α and β chains connecting the ECM to the cellular cytoskeleton (Hemler, 1998; Ivaska and Heino, 2000). Fibronectin is another widely expressed glycoprotein that contains an arginine-glycine-aspartate (RGD) cell attachment domain and binding sites for heparin, fibrin, and collagen (Pierschbacher and Ruoslahti, 1984). The RGD cell attachment domain can be bound with integrin to facilitate focal adhesion formation, which

is necessary for cell migration and therefore modulate cell migration (Clark et al., 2005; Leiss et al., 2008).

1.3 Intestinal development

Intestinal growth and development is critical for broiler health and performance (Noy and Sklan, 1998). With healthy gut growth and development, nutrients can be more efficiently absorbed in the small intestine, transported through the circulatory system to the liver, processed in the liver, and incorporated into muscle cells (Hocquette et al., 1998), which could result in a better muscle growth and potentially reduce the severity of WB.

1.3.1 Intestinal structure and function

Small intestine is an essential part of the digestive system where broilers digest and absorb nutrients. It is composed of three sections including the duodenum, jejunum, and ileum. The duodenum is commonly considered as the intestinal part from the ventricululs to pancreatic and bile ducts (Turk, 1982). The digestion process of the intestine starts in the duodenum with the acidic contents from the gizzard mixing with bile and pancreatic juices (Duke, 1986). The duodenum is short in length and most of lipid is digested here (Sklan et al., 1975). Intestinal midsection from the ducts to Meckels's diverticulum is called the jejunum (Duke, 1986). Nutrients including lipid, starch, and protein are mostly digested by the end of the jejunum. Ileum is the final intestinal section, which is from the diverticulum to ileo-caeco-colic junction (Duke, 1986). The empty weight of the ileum is lower than jejunum while the length is similar with the jejunum (Mitchell and Smith, 1991).

The remaining nutrients such as lipid, starch, protein, water, and mineral are digested and absorbed in ileum. Retention time through this section is much longer than the duodenum and jejunum (Weurding et al., 2001).

The intestine is a long tube made up of several layers (Figure 1.5) including a serosal layer, a longitudinal muscle layer with muscle fibers running along the length of the intestine, a circular muscle layer which is two times thicker than the longitudinal muscle layer, a submucosal layer, and a mucosal layer (Turk, 1982). The mucus is highly folded resulting in a number of folds which are finger-like projections extending into the intestinal lumen. These folds are called the villi and result in larger absorption surface area for higher absorptive ability (Iji et al., 2001). The surface of the villi is covered with epithelial cells, which line the inner surface of the intestines. The crypts are between the villi consisted of short glands.

1.3.2 Intestinal growth and development

Small intestine has a higher growth rate than the body weight immediate posthatch (Katanbaf et al., 1988). The proportional weight of the small intestine relative to body weight at 17 days of embryonic age is 1% and is increased to 3.5% at hatch (Uni et al., 2003b). The proportional intestinal weight peaks at 6 to 10 days of age (Sklan, 2001). Meanwhile, the intestinal length is increased by two to four times from hatch to 12 days of age (Uni, 2006). The intestinal growth is dependent on the stem cells in the crypts, which give rise to enterocytes (Cheng and Leblond, 1974). The enterocytes migrate up the villi and undergo morphological and functional changes (Sklan, 2001). All intestinal

enterocytes are proliferating at hatch while the number of proliferating enterocytes is decreased to around 50% at 3 days posthatch (Quaroni, 1985). The enterocytes shape changes in intestinal growth and development. The intestinal enterocytes are round and non-polar at hatch and become polar with a defined brush border at 2 days posthatch (Uni, 2006). Each villi is observed with one crypt in birds at hatch while three to four crypts are found per villi at 14 days post hatch (Uni et al., 2000).

Enzymes at the brush border of the enterocytes are secreted increasingly in the first two weeks posthatch for the final stage of digestion (Marchaim and Kulka, 1967). Enzyme involved in carbohydrate digestion such as sucrase is increased expressed at a considerable level just before hatch and continually increased in expression to the immediate posthatch period (Iji et al., 2001). For protein hydrolysis, enzymes such as aminopeptidase are expressed increasingly from 15 days of embryonic age until hatch and continues to increase in expression until after hatch (Uni et al., 2003b). Sucrase and aminopeptidase activities peak at 1 day of age (Iji et al., 2001; Uni et al., 2003b). Fat digestion is increased with age, with polyunsaturated fats more easily digested than saturated fats (Smulikowska, 1998). In addition, the enzyme activities vary with the intestinal sections. The duodenum has the lowest digestive capacity of disaccharides and the jejunum has the highest capacity with ileum being the intermediate (Uni et al., 1998). Enzymes associated with fat digestion are most effectively utilized in duodenum and jejunum (Johnson, 1992).

At the time of hatch, the intestinal mucosa of the broiler chicks is exposed to a number of antigens (Allen et al., 1993). The mucus layer serves as a barrier protecting the

intestinal epithelium from the attachment of pathogenic bacteria (Hill et al., 1990). Thus, mucosal development during incubation and growth plays a key role in broiler health. Mucin glycoproteins, especially mucin 2, make up the mucus layer in the small intestine. The mucins are produced by goblet cells at the base of crypts (Cheng and Leblond, 1974). Goblet cell population increases from 18 days of embryonic age to 7 days of age posthatch (Uni et al., 2003a). After the first week posthatch, the goblet cells number is stabilized (Smirnov et al., 2004). It is reported that mucins have the lowest expression in the duodenum and have a gradual increase in expression along the small intestine (Uni, 2006).

Transportation from hatchers to producers results in a lag time between hatch to initial access to feed, which will affect broiler intestinal development (Henderson et al., 2008). Effects of nutrition early posthatch on intestinal development have been investigated in various studies, suggesting that late access to feed delays intestinal development (Noy et al., 2001; Batal and Parsons, 2002; Ao et al., 2012; Jha et al., 2019). Reduced villus height and enterocytes number can be produced due to delayed feeding (Yamauchi et al., 1996). In contrast, enhanced intestinal development with improved intestinal morphology has been observed with sufficient early nutrient supply (Uni and Ferket, 2003). Since the first week posthatch is important for intestinal development and broiler chicks are sensitive to early nutrition, nutritional strategies are highly likely to improve gut health and nutrient absorption during the first week posthatch. With good nutrient absorption and transportation functions, nutrients can be utilized better in muscle

growth and development, which could decrease the development of the myopathies like WB.

1.4 Meat myopathies

Meat-type broilers have undergone dramatic genetic changes in growth performance and meat yield during the last decades (Figure 1.6). With the demand for poultry meat among consumers continuously increasing due to its low fat, high protein, affordable price and convenience in cooking (Petracci et al., 2015; Mottet and Tempio, 2017), broilers are selected for improved feed efficiency and higher breast meat yield (Brewer et al., 2012; Chatterjee et al., 2019). By 1920, chicks were grown in a small population size without specific breed for egg or meat production. Poultry meat was sold as a whole carcass in small markets. In the mid 1920s, commercial broilers began to be developed by cross breeding to improve growth performance. Currently, modern farms are mostly intensive high-yield farming. Market demand for cut-up portions has shifted selection from growth performance to meat yield such as the breast and legs (Barbut, 2015). Breed selection has resulted in increased feed efficiency with a 4.70 feed to meat gain changing to 1.80 (National Chicken Council, 2020). In 1925, chicks raised to a market weight of 1.13 kg required 112 days. In 2019, only 47 days are needed for growing a broiler to 2.87 kg. Poultry meat production has increased by more than four times from 1970 to 2019 (National Chicken Council, 2020).

1.4.1 Novel broiler myopathies

Selection for rapid growth and high meat yield has been accompanied by various meat myopathies such as WB (Sihvo et al., 2014; Velleman and Clark, 2015; Kuttappan et al., 2016; Tasoniero et al., 2016), white striping (WS) (Lorenzi et al., 2014; Mazzoni et al., 2015; Russo et al., 2015), stringy-spongy (SS) (Baldi et al., 2018; Tasoniero et al., 2020), pale, soft, exudative (PSE) (Barbut, 1997; van Laack et al., 2000; Woelfel et al., 2002; Zhu et al., 2012), and deep pectoral myopathy (DPM) (Richardson et al., 1980; Wight and Siller, 1980; Harpper et al., 1983; Siller, 1985). These myopathies have resulted in serious economic loss due to product downgrades and lack of palatability.

a. Wooden Breast

Wooden Breast is one of the primary myopathies which has been reported worldwide within the poultry industry (Sihvo et al., 2014; Kuttappan et al., 2016; Tasoniero et al., 2016). Its incidence vary between 48% and 85% (Kuttappan et al., 2017; Sihvo et al., 2017). Wooden Breast (Figure 1.7) is phenotypically characterized by palpation of a rigid p. major muscle (Sihvo et al., 2014). Presence of gelatinous fluid and scattered blood spots on the surface of the breast muscle associated with inflammatory signs are sometimes found in severe WB, which are required to remove by trimming (FSIS Notice, 2018). Histologically, WB has been identified with myodegeneration along with moderate or severe myofiber necrosis (Papah et al., 2017), fibrosis (Figure 1.8; Sihvo et al., 2014; Velleman and Clark, 2015), and inflammatory cell accumulation (Figure 1.9; Sihvo et al., 2014, 2017).

Not only histological changes but also meat quality reduction has been found in WB affected muscles. It is shown that WB affected tissues have higher lipid and lower protein contents (Soglia et al., 2016). The protein content in WB affected muscle is decreased to 21.7% from 23.0% in normal breast muscle. Collagen level associated with the hardness of the p. major muscle is increased in p. major muscle, which leads to reduced tenderness of the meat (Tasoniero et al., 2016; Velleman et al., 2017). Additionally, WB affected breast muscle has a higher pH value, lower water holding capacity, a higher drip loss and cooking loss (Mudalal et al., 2015; Brambila et al., 2017).

b. White striping

White stripping is characterized by white striation primarily in p. major muscles (Kuttappan et al., 2012). It is estimated that around 50% of breast muscles are affected by WS with 20%-30% have severe WS (Lorenzi et al., 2014; Russo et al., 2015). Histological changes such as intramuscular fat and connective tissue parallel to muscle fibers were observed in WS affected tissues (Mazzoni et al., 2015). In addition, some WS histological characteristics including necrosis and myodegeneration are overlapped with WB (Kuttappan et al., 2013b). The p. major muscles with severe WS had higher fat and collagen content, lower protein content, and higher pH (Petracci et al., 2013, 2014; Mazzoni et al., 2015). Higher cooking loss, drip loss, and b* (yellowness) occurred in WS condition (Tasoniero et al., 2016; Tijare et al., 2016). The incidence and severity of WS is closely associated with broiler weight with heavier birds having a higher chance to have WS (Kuttappan et al., 2013a).

c. Stringy-spongy

Stringy-spongy, also called a spaghetti meat, is characterized by loss of integrity of p. major muscle, which results in loose structure with separated muscle fiber bundles (Baldi et al., 2018). The SS is associated with a higher pH, increased moisture content, reduced protein content, decreased collagen content, and a reduced water holding capacity (Baldi et al., 2018; Tasoniero et al., 2020). After cooking, SS is soft due to the low collagen content (Baldi et al., 2019).

d. Pale, soft, exudative

The PSE condition is characterized by pale color, soft texture, and poor water holding capacity in broilers (Barbut, 1997; van Laack et al., 2000; Wilhelm et al., 2010). The PSE affected tissues usually have increased l* (lightness), declined pH, lower water holding capacity, and higher cooking loss (van Laack et al., 2000; Woelfel et al., 2002; Zhu et al., 2012). The occurrence of PSE is associated with genetics and environmental stress such as stress during transportation or slaughter processing (Mckee and Sams, 1998; Fletcher, 2002). Due to the mutation in the calcium channel gatekeeper proteins (α- and β-ryanodine receptors, RYR), calcium ions are accumulated in sarcoplasmic reticulum, which accelerates enzymes activities leading to protein degradation and muscle damage (Marchi et al., 2009; Oda et al., 2009; Wilhelm et al., 2010).

e. Deep pectoral myopathy

Deep pectoral myopathy is also known as green muscle disease. It was first discovered in turkeys (Dickinson et al., 1986) but most modern turkeys do not have DPM

due to genetic selection (Barbut, 2019). Only a small number of DPM cases have been reported in modern commercial broilers (Bianchi et al., 2006) with the prevalence much lower than WB, WS or SP. The DPM is a degenerative myopathy characterized by necrosis in supracoracoideus or pectoralis minor (p. minor) muscle and sometimes has a greenish discolored appearance (Richardson et al., 1980; Wight and Siller, 1980; Siller, 1985). Deep pectoral myopathy affected p. minor muscle has a rigid sheath made up of fibrous connective tissue, which restricts blood circulation and inhibits blood supply flowing into muscle (Wight and Siller, 1980). In addition, the increased muscle size due to selection for higher breast muscle mass results in intramuscular pressure occluding the blood vessels, leading to the muscle necrosis and degenerative changes (Harpper et al., 1983; Siller, 1985).

1.4.2 Wooden Breast plausible etiologies

Establishing a basic understanding of the onset and development of WB is critical for developing practical nutrition and management interventions to reduce the incidence and severity of WB. Although the exact etiology of WB remains unknown, several plausible etiologies have been suggested including oxidative stress, inflammation, and dysregulation of lipid deposition.

a. Oxidative stress

Wooden Breast affected broilers are under severe oxidative stress, which could be contributed to insufficient vascularization associated with selection for rapid growth (Mutryn et al., 2015). Excessive hypertrophy due to giant muscle fibers from selection

restricts connective tissue area and circulatory supply in heavy weight fast growing broilers (Dransfield and Sosnicki, 1999; Velleman et al., 2003). This will result in metabolic waste product accumulation and impaired oxygen supply (Velleman et al., 2003). The p. major muscle is mainly made up of fast twitch glycolytic (type IIB) fiber that metabolizes glycogen and glucose to lactate. Lactate cannot be removed by the impaired blood supply system and thereby is retained in muscle leading to muscle damage in broilers (Velleman et al., 2003; Mutryn et al., 2015). In addition, when oxygen is not reduced completely, ROS that has powerful oxidizing ability can be produced. If ROS is not removed properly by the antioxidant system, there is an imbalance between oxidants and antioxidants, which is referred to as oxidative stress (Voljč et al., 2011; Panda and Cherian, 2014).

Significant differences in the expression of genes involved in oxidative stress response in WB affected broilers have been identified in several RNA-sequencing studies (Mutryn et al., 2015; Abasht et al., 2016; Brothers et al., 2019). In terms of metabolomics study, Abasht et al. (2016) found numerous biological markers associated with oxidative stress in WB affected tissues that strongly suggests the association between WB and oxidative stress. They have also identified upregulated metabolites influencing redox homeostasis. The disruption of redox homeostasis will further result in altered ROS signaling and oxidative stress.

b. Inflammation

Initial muscle fiber degeneration is initiated with disruption of the sarcolemma (Straub et al., 1998). As the membrane integrity is impaired, calcium will influx from the

sarcoplasmic reticulum activating various calcium related pathways, which negatively alter the cellular functions (Dargelos et al., 2008). In response to abnormal cellular activities, immune cells such as heterophils are recruited to clean the damaged muscle fibers (Chazaud, 2016). Meanwhile, inflammatory cytokines such as interleukin-1β are secreted recruiting more immune cells such as macrophages (Brigitte et al., 2010). Continuous infiltration of these immune cells and inflammatory cytokines contribute to the inflammation of the breast muscle (Chazaud, 2016). Since inflammation is harmful for muscle regeneration, the damaged muscle fibers cannot be fully repaired through regeneration process and will be replaced with connective tissue resulting in fibrosis associated with WB (Sihvo et al., 2014).

Inflammation in WB affected breast muscle has been suggested by histological changes showing infiltration of inflammatory cells (Sihvo et al., 2014), necrosis and lysis of fibers (Velleman and Clark, 2015; Papah et al., 2017). Metabolomics analysis confirmed the existence of inflammation in WB affected p. major muscle because of increased levels of inflammatory metabolites (Abasht et al., 2016). Zambonelli et al. (2017) identified differentially expressed genes involved in inflammatory responses in WB broilers.

c. Dysregulation of lipid metabolism

Recently, dysregulated lipid metabolism has been closely associated with WB. Histological evidence of excess lipid accumulation in p. major muscle indicates the dysregulation of lipid metabolism (Papah et al., 2017). Metabolomic analysis (Abasht et al., 2016) and RNA-sequencing studies (Brothers et al., 2019; Lake et al., 2019) are in

agreement with the microscopic changes that metabolites and genes such as *peroxisome proliferator-activated receptor gamma* (*PPARγ*) and *CCAAT/enhancer binding protein alpha* (*CEBPα*) involved in altered glucose utilization are differentially expressed in WB affected muscles. Dysregulation of glucose utilization is suggested by decreased levels of glycolytic intermediates and end products (Abasht et al., 2016). Inhibition of glycolysis is accompanied by increased glucose uptake through alternative metabolic pathways such as pentose phosphate (Abasht et al., 2016; Papah et al., 2018). Although glucose is utilized by breast muscle, it is not fully used for producing energy as in normal broilers, resulting in excess lipid accumulation and fibrosis in p. major muscle (Lake and Abasht, 2020). Moreover, lipid accumulation can lead to an immune response (Lee and Hwang, 2006) and higher ROS production (Goglia and Skulachev, 2003) contributing to inflammation and oxidative stress. These will further exaggerate the severity of WB.

1.5 Early posthatch nutritional interventions

Posthatch muscle growth is dependent on satellite cells that have the highest mitotic activity the first week posthatch (Halevy et al., 2000). Satellite cells are multipotential stem cells which means they can follow various developmental pathways forming the cellular lineage for skeletal muscle, adipocytes, and chondrocytes due to changes in nutritional supplementation (Asakura et al., 2001; Shefer et al., 2004). In this way, nutritional strategies during early posthatch period can be used to modify satellite cells activities and thereby influence the muscle structure. Since WB is associated with oxidative stress

(Mutryn et al., 2015; Abasht et al., 2016; Brothers et al., 2019) and inflammation (Mutryn et al., 2015; Zambonelli et al., 2017), early posthatch nutritional interventions to reduce oxidative stress and inflammation will likely decrease the incidence and severity of WB myopathy.

1.5.1 Vitamin E

Vitamin E is a powerful fat-soluble antioxidant protecting cells and tissues from oxidative damage through scavenging free radicals (Voljč et al., 2011). Broilers cannot produce VE on their own. Thus, VE supply for the broilers is dependent on their diets (Hässig et al., 1999). There are eight forms of VE including four tocopherols and four tocotrienols. Among these eight forms, α-tocopherol is commonly used in poultry industry as it is retained by the body (Panda and Cherian, 2014). It has been reported that α-tocopherol supplementation significantly enhances muscular and intestinal α-tocopherol retention and improved antioxidant capacity both in the muscle (Cheng et al., 2016) and intestine (Cheng et al., 2018) in broilers. The antioxidant activity is through VE donating electrons to free radicals, which reduces combination of free radicals and fatty acids in cell membrane (Sakamoto et al., 2006). The integrity of the cell membrane is thereby maintained well through preventing membrane phospholipids oxidation (Bou et al., 2009).

The recommended VE requirement for broilers in National Research Council (NRC, 1994) is 10 mg/kg of the diet. However, the latest NRC was revised in 1994. As the weight of broilers at market age has increased 35% since 1994 (National Chicken Council, 2019), the NRC may not meet the current VE requirement for modern broilers. Higher

levels of oxidative stress may require higher level of VE. Studies have indicated that supplementation of 200 mg/kg of VE was able to reduce oxidation in broilers (Rebolé et al., 2006; Taulescu et al., 2011).

1.5.2 Omega-3 fatty acids

Omega-3 polyunsaturated fatty acids (PUFA) are characterized by a structure with a double bond distributed three atoms away from the terminal methyl group. There are three types of n-3 fatty acids including α-linolenic acid (18:3n-3), eicosapentaenoic acid (20:5n-3; EPA), and docosahexaenoic acid (22:6n-3; DHA) (Reiser and Gibson, 1950). Omega-3 fatty acids are known to have anti-inflammatory properties (Simopoulos, 2002; Calder, 2003; Yu et al., 2018) by the synthesis of oxylipins from PUFA called eicosanoids (C20-derived) or docosanoids (C22-derived) (Calder, 2003). Eicosanoids are involved in various of inflammatory activities. They are commonly synthesized by arachidonic acids (C20:4n-6) (Brock and Peters-Golden, 2007). Supplementation of n-3 fatty acids decreases the arachidonic acids levels and therefore reduces the contents of n-6 derived eicosanoids that are produced from arachidonic acids (Calder, 2006). Changing dietary n-6/n-3 ratio can influence eicosanoid profiles because increased dietary n-3 PUFA can alter n-6 eicosanoids production by increasing n-3 derived oxylipins as the precursor PUFA (n-6 or n-3) utilize the same enzymes for synthesis. The n-3 derived eicosanoids are less biologically potent than eicosanoids synthesized from arachidonic acids and have anti-inflammatory effects (Calder, 2012). In this way, n-3 fatty acids exert anti-inflammatory effects.

Poultry has to intake PUFA from their diets as they cannot synthesize PUFA (Reiser and Gibson, 1950). The n-6/n-3 ratio in commercial broiler diets is usually over 30:1 having a very low n-3 fatty acids level (Cherian, 2008). To increase n-3 fatty acids level, fish oil can be added in the diet as it contains high level of EPA and DHA (Bharath et al., 2017). Currently, there is no recommended n-6/n-3 ratio for poultry diets in NRC. However, it has been hypothesized that a ratio of 1:1 to 4:1 of n-6/n-3 ratio in diet can improve human health (Simopoulos, 2002). Additionally, supplementation of n-3 fatty acids increases fatty acids unsaturation resulting in higher potential of lipid oxidation. Thus, the combination of VE and n-3 fatty acids can prevent oxidative stress while reducing inflammation (Taulescu et al., 2011).

1.5.3 Alpha lipoic acid

Alpha lipoic acid is both fat and water soluble short chain fatty acid that can be absorbed and transported efficiently across cell membranes (Kofuji et al., 2008). It has strong antioxidant effects by minimizing free radical activities (El-Senousey et al., 2013). After being absorbed in the intestine, ALA is rapidly reduced into dihydrolipoic acid (DHLA) (Mackenzie et al., 2006). The DHLA acts as an antioxidant scavenging free radicals such as hydroxyl, peroxyl, and superoxide radicals (Packer et al., 1996). Moreover, activities of antioxidant enzymes such as glutathione peroxidase and superoxide dismutase can be improved contributing to lipid stabilization (Srilatha et al., 2010). Anti-inflammatory effects have also been found in that ALA can inhibit pro-inflammatory cytokines (Li et al., 2014). El-Senousey et al. (2018) reported that 500 mg/kg ALA showed

a beneficial effect on reducing oxidative stress as well as inflammation in broilers. Additionally, VE is suggested to have synergistic effect with ALA (Figure 1.10) that ALA helps recycle VE from its oxidized form (Sohaib et al., 2018). The reduced form of ALA, DHLA, can recycle ubi-semiquinone, semi-dehydroascorbate radical, glutathione disulphide, and thloredoxin, contributing to regeneration of VE (Sohaib et al., 2018).

1.6 Hypothesis and objectives of the research

Breast muscles affected with WB are under severe oxidative stress and inflammation. Although previous studies have identified the antioxidant property of VE and ALA, and the anti-inflammatory effect of n-3 fatty acids, there are no publications using VE, n-3 fatty acids, and ALA to reduce WB or determining the relationship between gut health and WB. Thus, the overall hypothesis of the current study was that WB incidence and severity is influenced by nutritional strategies used during the early posthatch period. The overall objective of this study was to reduce the incidence and severity of WB myopathy through early posthatch nutritional interventions including vitamin E (VE) and alpha lipoic acid (ALA) with antioxidant properties, and omega-3 (n-3) fatty acids with anti-inflammatory effects.

Accordingly, the five specific aims studied were (Figure 1.11):

Aim 1 was to identify the effects of dietary VE, n-3 fatty acids, and combining both during starter and grower phase on growth performance, meat quality and severity of WB myopathy. It was hypothesized that the dietary treatments with antioxidant and anti-

inflammatory properties during the period of maximal satellite cell activity would influence meat quality and the incidence and severity of WB myopathy.

Aim 2 was to identify the effects of dietary VE, n-3 fatty acids, and combining both during starter and grower phase on breast muscle morphological structure and gene expression in the breast muscle. The hypothesis was that the dietary treatments targeting reducing oxidative stress and inflammation during the early posthatch period would affect breast muscle growth properties and alter oxidative stress and inflammation status in muscle.

Aim 3 was to identify the effects of dietary VE, n-3 fatty acids, and combining both during starter and grower phase on intestinal morphological structure and gene expression in the broilers. The hypothesis was that the dietary treatments early posthatch would influence intestinal growth as well as alter nutrient absorption, oxidative stress and inflammation status in small intestine.

Aim 4 was to evaluate the effects of dietary VE, ALA, and combining both on the developmental onset, severity, and progression of the WB myopathy during the early posthatch period based on developmental changes in muscle morphological structure and gene expression in broilers. It was hypothesized that nutritional strategies with antioxidant and anti-inflammatory activities would influence WB development early posthatch and affect breast muscle growth and development.

Aim 5 was to evaluate the effects of dietary VE, ALA, and combining both on the intestinal developmental changes in ileal morphological structure and gene expression

during the early posthatch period. The hypothesis was that early posthatch nutritional interventions would influence intestinal growth and development and affect inflammation status in small intestine.

Specifically, the first aim of the study was to identify the effects of dietary VE and n-3 fatty acids independently or in combination when fed during the starter phase (0 to 10 day) or grower phase (11 to 24 day) on growth performance, meat yield, meat quality, and severity of WB myopathy. A total of 210 Ross 708 broiler chicks were randomly assigned into seven experimental groups with 10 replicates of 3 birds each. The control group was fed with corn-soybean meal basal diet with VE (10 IU /kg) and n-3 fatty acids (n-6/n-3 ratio of 30.2:1) at a standard level during the entire study (0 to 58 day). Supplementation of 200 IU/kg of VE (200 IU/kg), n-3 fatty acids (n-6/n-3 ratio of 3.2:1), or combination of both was performed during the starter phase or grower phase. Growth performance, meat yield, meat quality, and WB scores were obtained.

The second aim studied the effects of VE and n-3 fatty acids on the severity of WB, morphological structure of the p. major muscle, and expression of genes likely associated with WB. The p. major muscle samples were collected from aim 1 for p. major muscle morphology evaluation and gene expression analysis. Morphological assessment of the p. major muscle included myofiber width, perimysial and endomysial connective tissue space, overall morphological structure, and scoring of WB microscopically. Gene expression associated with muscle development and growth factors, inflammation, ECM, and glucose metabolism was measured using Nanostring analysis.

Since WB affected birds are under severe inflammation, which is a complex process that is often systemic influencing many physiological systems throughout the entire body (Chawla, 2011), inflammation in intestine may be closely associated with the development of WB. Thus, the third aim further identified the effects of VE, n-3 fatty acids, and combination of both during the starter phase (0 to 10 day) or grower phase (11 to 24 day) on intestinal morphology and expression of genes associated with gut health and nutrient transport, and to identify the relationship between gut health and WB. Intestinal samples were collected from aim 1 for ileal morphological assessment and gene expression analysis. Villus height, crypt depth, villus width, distance between villi, and number of intraepithelial lymphocytes (IEL) were obtained. Expression of 21 genes associated with gut health and nutrient transport was measured using Nanostring analysis. To analyze correlation between gut health and WB, final body weight and p. major muscle weight from aim 1, and p. major muscle morphology and gene expression from aim 2 were used for correlation coefficients analysis with ileal morphology and gene expression.

Wooden Breast has been identified in broilers as early as 18 days of age and the severity of WB increases with age and growth of the birds (Sihvo et al., 2017). Vitamin E and ALA are two nutrients that have powerful antioxidant capacities (El-Senousey et al., 2018). Therefore, the objective of the fourth aim was to identify the effects of VE and ALA on the developmental onset, severity, and progression of WB based on p. major muscle morphology and expression of genes associated with WB in broilers during the first 3 week posthatch. A total of 160 newly hatched Ross 708 broiler chicks were randomly assigned

into a control group and three dietary treatments with 10 replicates of four birds each. Supplementation of VE (160 mg/kg) and ALA (500 mg/kg) independently and in combination were fed during the first 3 weeks posthatch. At 1, 2 and 3 weeks of age, one chick from each pen was harvested. Growth performance and phenotypic WB score were obtained. Microscopic assessment of p. major muscle included myofiber width, perimysial/endomysial connective tissue spacing, morphology score, and microscopic WB score. Expression of genes associated with muscle formation and growth, adipogenesis, ECM, oxidative stress and inflammation were measured by real-time quantitative PCR.

The fifth aim was to evaluate VE and ALA effects on intestinal developmental changes in ileal morphology and expression of genes related with gut nutrient transport, barrier integrity, and inflammation in broilers during the first 3 weeks posthatch. Intestinal samples were collected from aim 4 for intestinal morphology evaluation and gene expression analysis. Plasma VE concentration and ileal morphology were determined. Expression of genes related with gut nutrient transport, barrier integrity, and inflammation was measured by real-time quantitative PCR.

Taken together, WB myopathy has arisen in the breast muscle of modern commercial broilers due to selection for higher growth rate and breast muscle yield (Brewer et al., 2012; Chatterjee et al., 2019). The WB affected muscles are palpably hard (Sihvo et al., 2014) under severe oxidative stress (Mutryn et al., 2015; Abasht et al., 2016; Brothers et al., 2019) and inflammation (Mutryn et al., 2015; Zambonelli et al., 2017). The development of WB is involved with skeletal muscle and small intestinal growth. Early

posthatch nutritional interventions beneficial for muscle and intestinal growth and development can be potentially used to reduce WB.

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Table 1.1 Properties of different muscle fiber types

	Type I (Slow-Oxidative)	Type IIa (Fast-Oxidative)	Type IIb (Fast-Glycolytic)
Contraction speed	Slow	Moderately fast	Fast
Capillarization	High	Intermediate	Low
Myoglobin content	High	High	Low
Glycolytic capacity	Low	Intermediate	High
Mitochondrial density	High	Intermediate	Low
Fiber color	Red (Dark)	Red	White

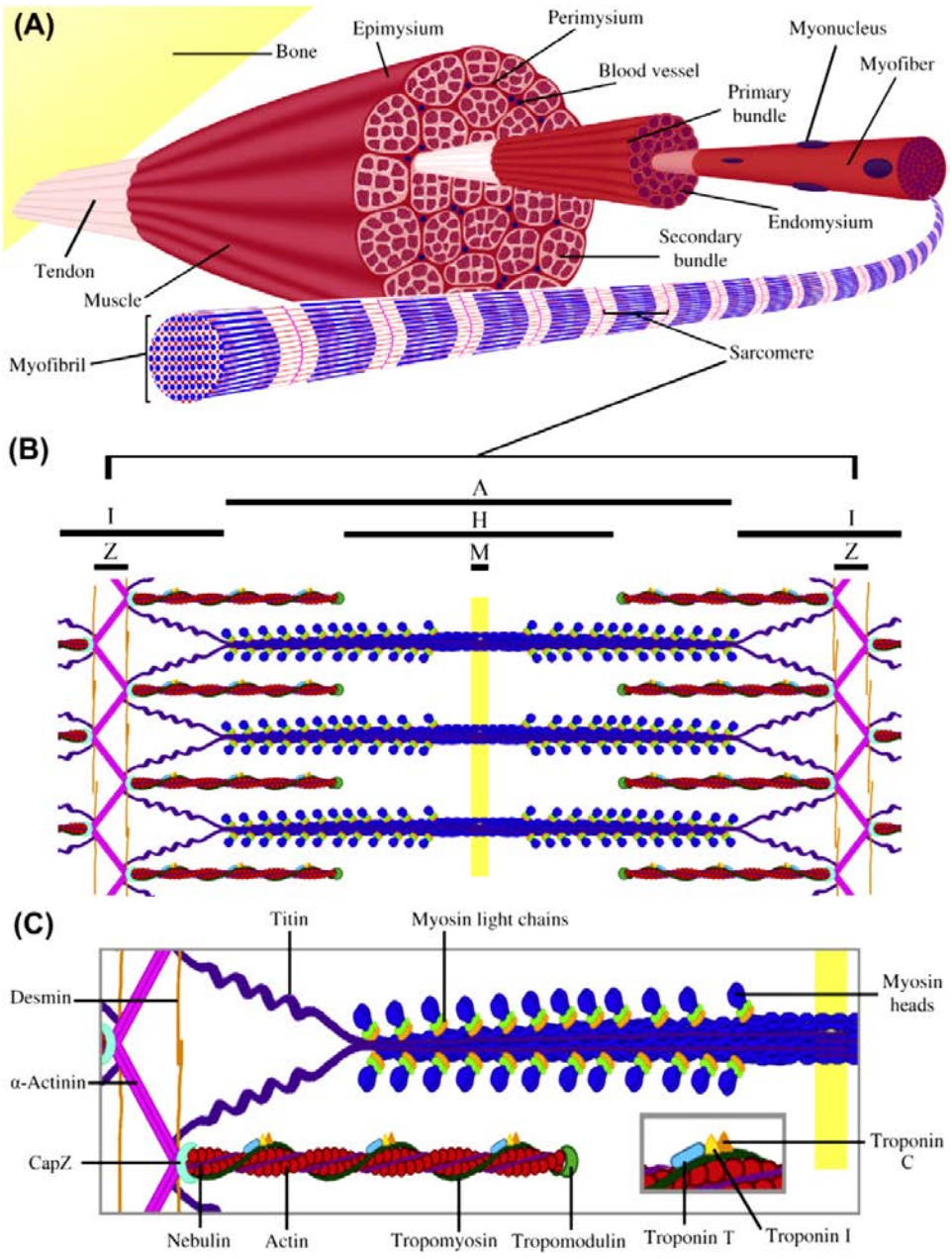

Figure 1.1 Skeletal muscle structure. (A) Overview of a cross-sectional area of muscle highlighting myofiber structure; (B) Sarcomere structure; (C) Myosin-actin overlap with the associated molecules. Adapted from Velleman and McFarland (2014).

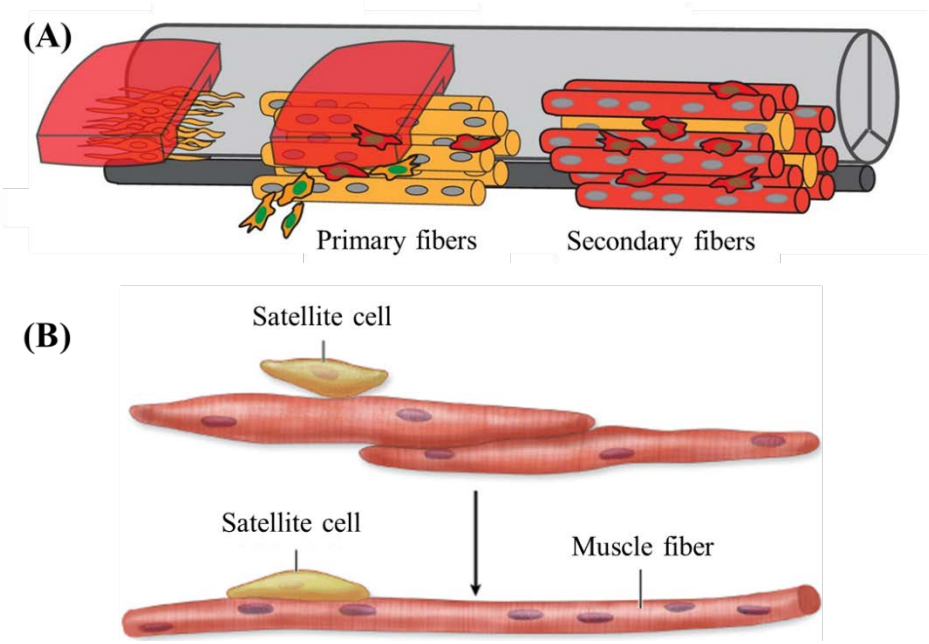

Figure 1.2 Schematic of skeletal muscle growth through hyperplasia and hypertrophy. There are two phases during skeletal muscle growth including the embryonic phase of fiber formation (A) and posthatch phase of muscle growth (B). In embryonic phase, mononucleated myoblasts fuse with the adjacent one to form primary muscle fibers. Stepwise formation leads to the formation of the secondary fibers. At the time of hatch, the myoblasts are withdrawn from the cell cycle and myofiber formation is complete. The embryonic formation of muscle fiber with muscle cell number increasing through proliferation is referred to as hyperplasia. Posthatch muscle growth is dependent on myogenic stem cells called the satellite cells. Satellite cells fuse with multinucleated myofibers, donate their nuclei, and increase protein synthesis capabilities. This results in muscle growth through hypertrophy or the enlargement of existing muscle fibers. Adapted from Chal and Pourquié (2017).

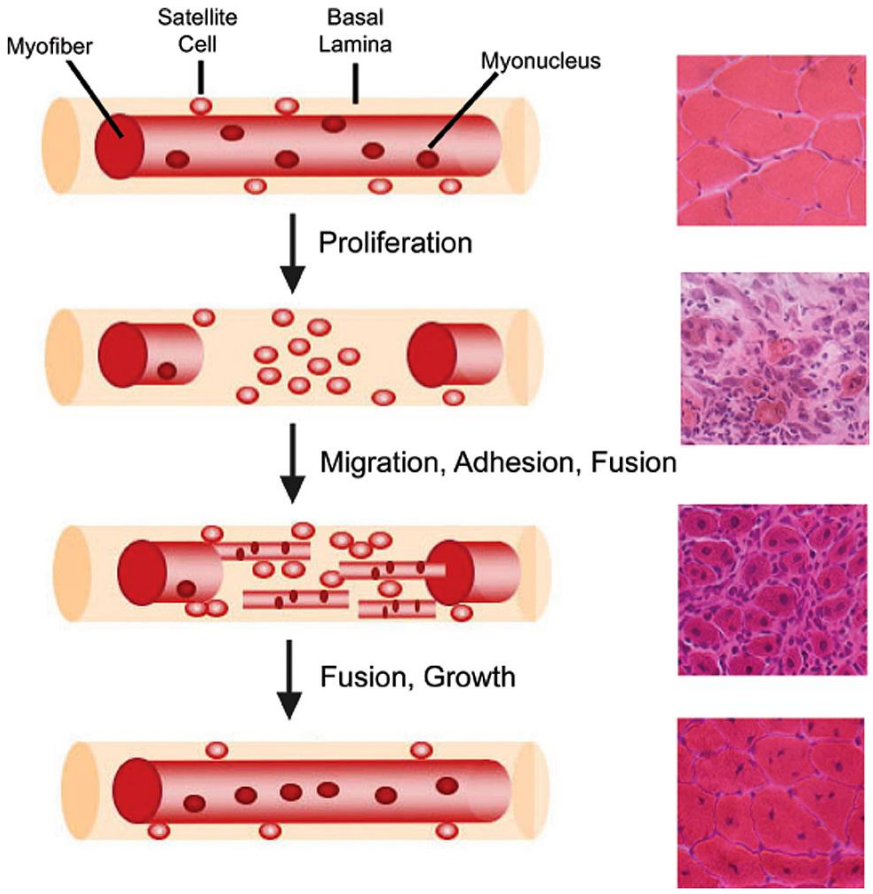

Figure 1.3 Schematic of skeletal muscle regeneration. If the muscle is damaged, satellite cells can be reactivated to undergo regeneration process repairing the damaged fibers or forming new muscle fibers when the muscle is damaged. The self-renewal of the satellite cells is required for generation of new muscle fibers to maintain satellite cell pool. The satellite cells attach to the existing muscle fibers and proliferate, fuse with the damaged muscle fibers or form new muscle fibers. Adapted from Randolph and Pavlath (2015).

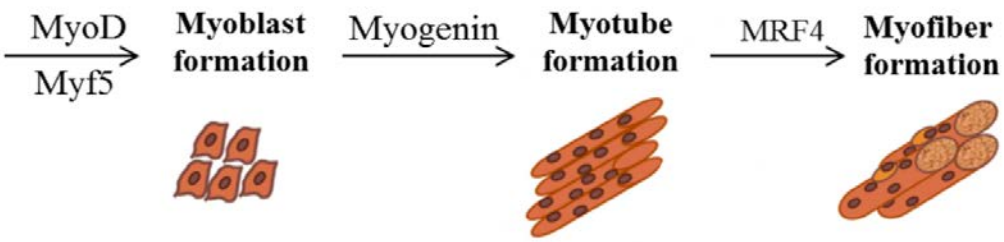

Figure 1.4 Myogenic regulatory factors associated with muscle growth and development. Muscle growth and development during both embryonic and posthatch phases requires the expression of myogenic regulatory factors. Myogenic determination factor 1 (MyoD) and myogenic factor 5 (Myf5) are necessary for myoblast formation and satellite cell proliferation. Myogenin is needed for multinucleated myotube formation. Muscle regulatory factor 4 (MRF4) is required for the myofiber formation.

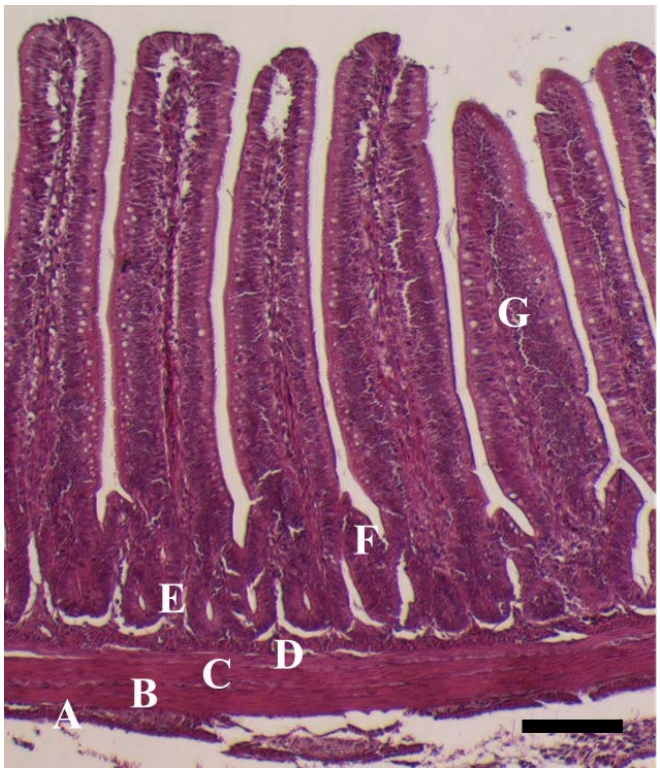

Figure 1.5 Intestinal structure. The intestine is a long tube made up of several layers including a serosal layer (A), a longitudinal muscle layer (B), a circular muscle layer (C), a submucosal layer (D), and a mucosal layer (E). F: Crypt; G: Villi. Scale bar = 200 μm.

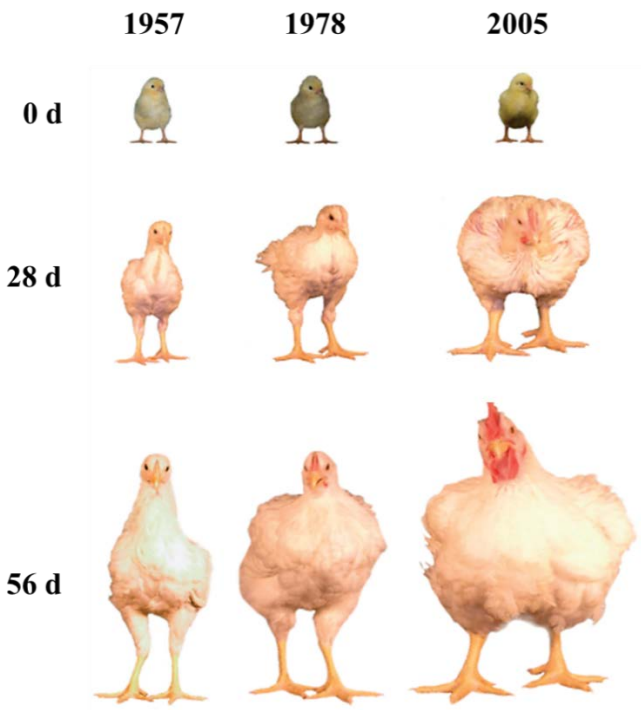

Figure 1.6 Development of meat-type broiler size with age. Meat-type broilers have undergone dramatic genetic changes in growth performance and meat yield during the last decades. Broilers in 1957 and 1978 were from University of Alberta Meat Control strains and in 2005 were Ross 308 line. Adapted from Zuidhof et al. (2014).

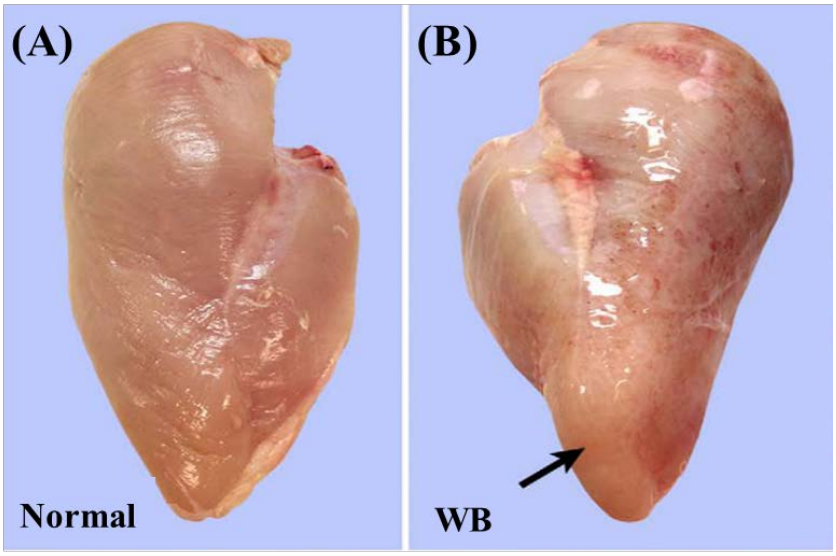

Figure 1.7 Comparison of normal pectoralis major (p. major) muscle (A) and Wooden Breast (WB) affected muscle (B) in broilers at 5 to 6 weeks of age. Wooden Breast affected p. major muscle is palpably hard, covered with gelatinous fluid and scattered blood spots on the surface of the muscle. The arrow shows the bulge at the caudal end of the muscle associated with WB. Adapted from Sihvo et al. (2014).

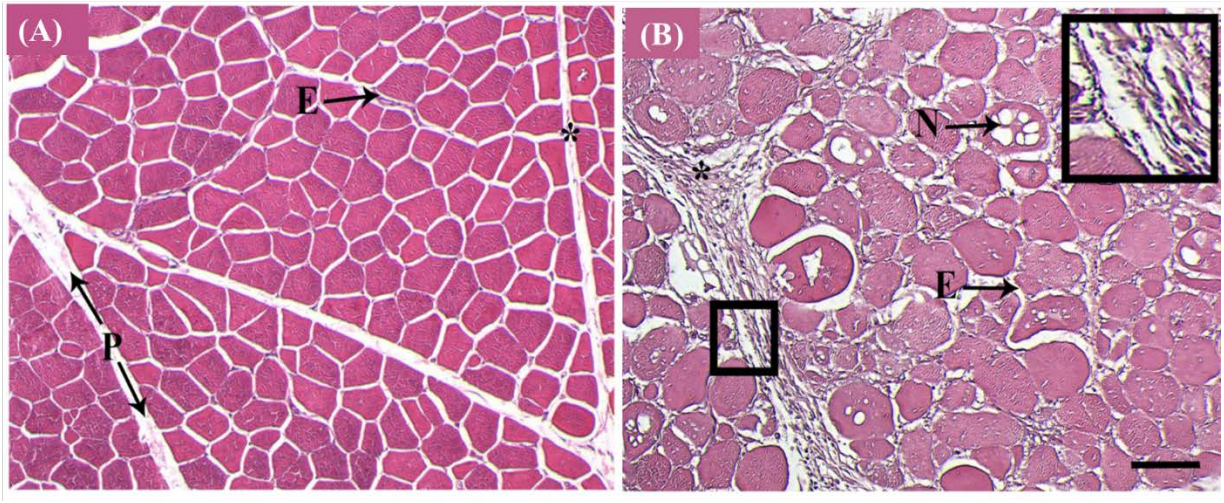

Figure 1.8 Representative photomicrographs of normal pectoralis major (p. major) muscle (A) and Wooden Breast (WB) affected muscle (B). Normal p. major muscle has distinct muscle fibers, perimysial and endomysial connective tissue spacin. Wooden Breast affected muscle has been identified with myodegeneration along with moderate or severe myofiber necrosis, fibrosis, and inflammatory cell accumulation. *: Collagen; P: Perimysial connective tissue; E: Endomysial connective tissue; N: Necrotic myofibers. The box contains the enlargement of the collagen. Scale bar = 100 μm.

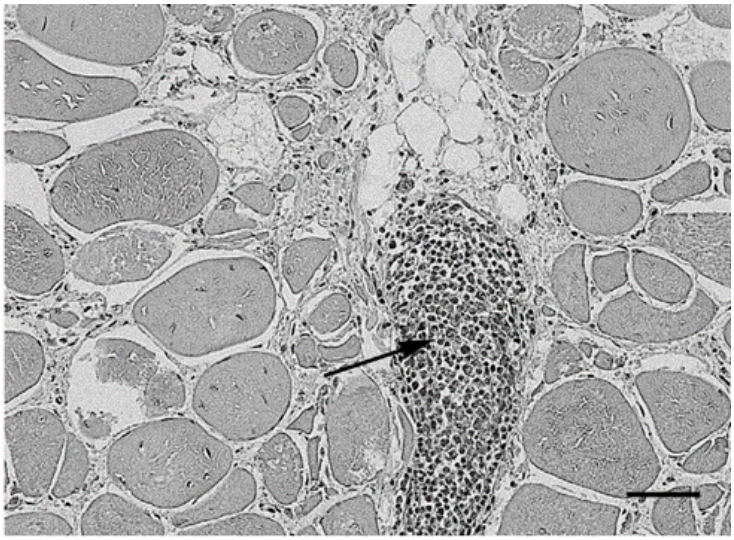

Figure 1.9 Macrophage infiltration associated with Wooden Breast myopathy. The arrow shows the infiltration of the macrophages. Scale bar = 100 μm. Adapted from Velleman and Clark (2015).

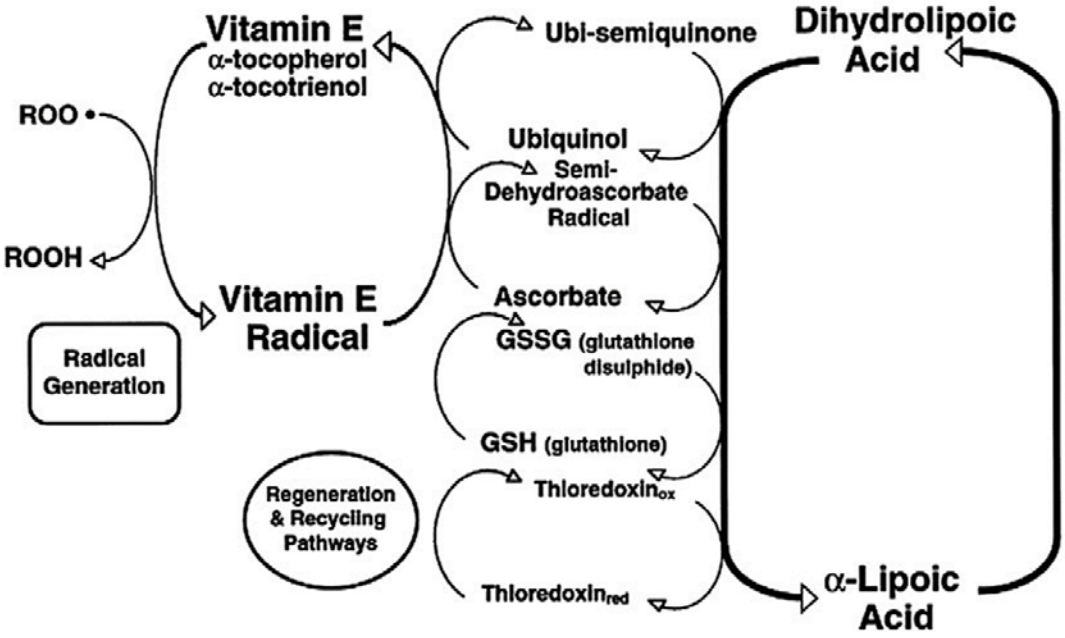

Figure 1.10 Action mechanism of alpha lipoic acid recycling vitamin E. Alpha lipoic acid is rapidly reduced into dihydrolipoic acid (DHLA) after being absorbed. The DHLA can recycle ubi-semiquinone, semi-dehydroascorbate radical, glutathione disulphide, and thloredoxin, contributing to regeneration of vitamin E. Adapted from Sohaib et al. (2018).

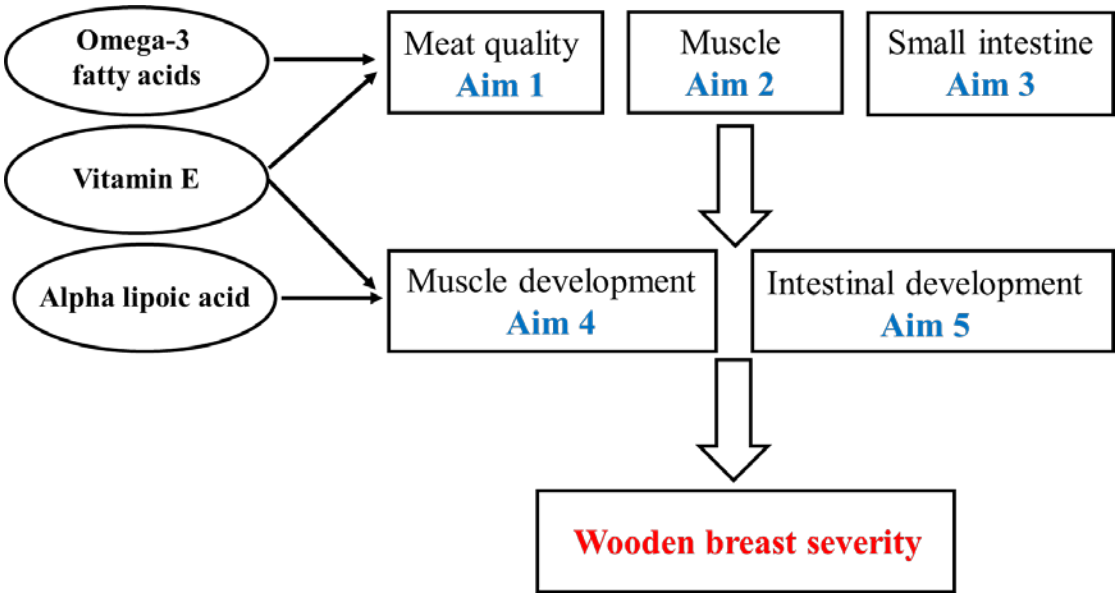

Figure 1.11 Schematic of the specific aims in the current study. Five specific aims were studied in the current study to address the overall objective of reducing the incidence and severity of Wooden Breast (WB) myopathy through early posthatch nutritional interventions. Aim 1, 2, and 3 were to evaluate the effects of dietary vitamin E (200 IU/kg), omega-3 fatty acids (n-6/n-3 ratio of 3.2:1), or combination of both during the starter (0 to 10 day) or grower phase (11 to 24 day) on severity of WB, meat quality, pectoralis major muscle structure, and small intestinal structure in broilers at market age (58 day). Aim 4 and 5 further evaluated the effects of vitamin E (160 mg/kg) and alpha lipoic acid (500 mg/kg) independently and in combination on muscle and small intestinal development associated with WB during the first three weeks posthatch.

Chapter 2: Effect of Vitamin E and Omega-3 Fatty Acids Early Posthatch Supplementaion on Reducing the Severity of Wooden Breast Myopathy in Broilers*

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Abstract

The Wooden Breast (WB) myopathy is identified by the palpation of a rigid pectoralis major (p. major) muscle and is characterized as a fibrotic, necrotic p. major muscle disorder in broilers resulting in reduced breast meat quality. Breast muscle affected with WB is under severe oxidative stress and inflammation. The objectives were to identify the effects of dietary vitamin E (VE) and omega-3 (n-3) fatty acids independently or in combination when fed during the starter phase (0 to 10 day) or grower phase (11 to 24 day) on growth performance, meat yield, meat quality, severity of WB myopathy, and to determine the most beneficial dietary supplementation period. A total of 210 Ross 708 broiler chicks were randomly assigned into seven experimental groups with 10 replicates of 3 birds each. The control group was fed with corn-soybean meal basal diet with VE (10 IU /kg) and n-3 fatty acids (n-6/n-3 ratio of 30.2:1) at a standard level during the entire study (0 to 58 day). Supplementation of 200 IU/kg of VE (200 IU/kg), n-3 fatty acids (n-

6/n-3 ratio of 3.2:1), or combination of both was performed during the starter phase or grower phase. Growth performance, meat yield, meat quality, and WB scores were obtained. There was no significant difference in final body weight and meat yield when VE was increased ($P > 0.05$). In contrast, n-3 fatty acids supplementation in starter diets significantly decreased final body weight, hot carcass weight, and chilled carcass weight of broilers ($P \leq 0.05$). The p. major muscle from broilers supplemented with VE in starter diets had lower shear force than in grower diets ($P \leq 0.05$). Supplemental VE reduced the severity of WB and in starter diets showed a more beneficial effect than those fed VE in the grower diets. These data are suggestive that additional supplementation of dietary VE may reduce the severity of WB and promote breast meat quality without adversely affecting growth performance and meat yield.

2.1 Introduction

The poultry industry has increased broiler production by selecting for growth, feed conversion, and muscle mass accretion including pectoralis major muscle (p. major) or breast muscle yield. Breast muscles from meat-type commercial broilers are 10 times larger than those from broilers marketed in 1955 at the same age (Collins et al., 2014). As a result of these selection practices, meat quality challenges (Petracci et al., 2013; Mazzoni et al., 2015; Tasoniero et al., 2016) and myopathies (Kuttappan et al., 2012b; Sihvo et al., 2014; Velleman, 2015) have arisen in the breast muscles of modern commercial broilers. Among the myopathies, Wooden Breast (WB) is of great concern to the poultry industry as it is

present within the broiler industry worldwide (Sihvo et al., 2014; Kuttappan et al., 2016; Baldi et al., 2018). This myopathy has created considerable economic losses for the industry due to product downgrades and negative consumer attitudes towards WB breast meat (Sihvo et al., 2014).

Wooden Breast is identified by the palpation of a rigid p. major muscle and has moderate or severe myodegeneration along with different levels of myofiber necrosis (Papah et al., 2017; Baldi et al., 2018), fibrosis (Sihvo et al., 2014; Velleman and Clark, 2015), and inflammatory cell accumulation (Sihvo et al., 2014, 2017). Presence of WB often coexists with White Striping (WS) (Abasht et al., 2016). White Stripping is characterized by white striation of intramuscular fat and connective tissue parallel to muscle fibers mainly in the p. major muscle (Kuttappan et al., 2012a). The WB myopathies not only lead to histological changes, but also to carcass and meat quality reduction. Studies have shown that WB affected tissues have lower water holding capacity (Petracci et al., 2013; Soglia et al., 2016; Sanchez Brambila et al., 2017) and decreased tenderness (Petracci et al., 2015; Tasoniero et al., 2016) compared with normal tissues. Nutritional values of WB affected muscle are significantly changed with higher moisture and lipid content (Petracci et al., 2014; Mazzoni et al., 2015).

Although the exact etiology of WB remains unknown, several studies have indicated that WB affected broilers are under severe oxidative stress (Mutryn et al., 2015; Abasht et al., 2016) and inflammation (Sihvo et al., 2014; Mudalal et al., 2015). Since posthatch muscle growth is dependent on myogenic stem cell called satellite cells, which

have their maximal activity during the first week posthatch (Mozdziak et al., 2002) and are sensitive to nutritional changes (Halevy et al., 2000; Powell et al., 2014; Velleman et al., 2014), early posthatch nutritional strategies to reduce oxidative stress and inflammation will likely decrease the incidence and severity of WB myopathies.

Among the numerous antioxidants, vitamin E (VE) is a very powerful lipid-soluble antioxidant that prevents oxidative damage in cells and tissue (Voljč et al., 2011). According to the National Research Council (NRC: National Research Council, 1994), the nutrient requirement of VE for broilers is 10 mg/kg of the diet. However, the latest NRC requirements were revised in 1994. According to the National Chicken Council (2019), the weight of broilers at market age has increased 35% since 1994. In addition, higher levels of oxidative stress as a result of rapid growth may require higher levels of VE. Therefore, the latest NRC guidelines may not reflect the current VE requirement for modern broilers.

Omega-3 (n-3) fatty acids are polyunsaturated fatty acids (PUFA) and have been found to exert anti-inflammatory effects through altering pro-inflammatory eicosanoids and cytokines profiles (Simopoulos, 2002; Calder, 2003; Rahimi et al., 2011; Yu et al., 2018). Linoleic acid (C18:2) and linolenic acid (C18:3) are dietary essential fatty acids for poultry (Reiser and Gibson, 1950). They are also the precursors for synthesis of very long-chain PUFA, arachidonic acid and eicosapentanoic acid, which are the precursors of inflammatory eicosanoids. Although the NRC has not given a recommended ratio of omega-6 (n-6)/n-3 in poultry diets, a ratio of 1:1 to 4:1 of n-6/n-3 in diets for humans are suggested to achieve optimal health benefits (Simopoulos, 2002). Commercial poultry diets

commonly use diets with n-6/n-3 ratio of over 20:1 and therefore contain very low contents of n-3 fatty acids (Cherian, 2008). The low n-3 fatty acids contents are from a high percentage of corn oil in the diets. As opposed to corn oil, fish oil is a well-known source that can enrich n-3 fatty acids (Bharath et al., 2017). However, a very high level of fish oil, especially when incorporated in finisher diets, could increase off flavors and oxidation in meat products (Lopez-Ferrer et al., 2001). Furthermore, supplemental VE could be added to obtain better immune function (Taulescu et al., 2011) and potentially reduce inflammatory damage associated with the onset of WB.

Although previous studies have found the antioxidant effect of VE (Voljč et al., 2011) and anti-inflammatory effect of n-3 fatty acids to be beneficial (Simopoulos, 2002; Calder, 2003; Rahimi et al., 2011; Yu et al., 2018), there are no published reports using VE and n-3 fatty acids to reduce the WB myopathy or determining the optimal period to administer VE and n-3 fatty acids. Thus, the objective of the present study was to identify the effects of increasing VE, n-3 fatty acids, and combination of both during the starter phase (0 to 10 day) or grower phase (11 to 24 day) on growth performance, meat yield, meat quality, and severity of WB myopathy.

2.2 Materials and Methods

2.2.1 Birds and Experimental Diets

All bird activities were approved by the Institutional Animal Care and Use Committee of The Ohio State University. A total of 210 commercial Ross 708 broiler

chicks were individually weighed, wing banded, and placed into pens immediately after hatch. Broilers had *ad libitum* access to feed and water. They were randomly divided into seven experimental groups with 10 replications of 3 birds each in a completely randomized design. The control group was fed a corn-soybean meal basal diet with VE (Dl-α-tocopherol acetate, 10 IU /kg) and n-3 fatty acids (n-6/n-3 ratio of 30.2:1) at a standard level during starter phase (0 to 10 day), grower phase (11 to 24 day), and finisher phase (25 to 58 day). Additional supplemental VE or n-3 fatty acids were fed during the starter or grower phase. For the starter dietary supplementation, starter VE, starter n-3, and starter VE and n-3 groups were fed with the basal diet supplemented at concentrations of 200 IU/kg diet of VE, n-3 fatty acids with a n-6/n-3 ratio of 3.2:1, or a combination of both. The grower and finisher diets were the same as the control group. For the grower dietary supplementation, grower VE, grower n-3, and grower VE and n-3 groups were fed the basal diets supplemented at concentrations of 200 IU/kg diet of VE, n-3 fatty acids with a n-6/n-3 ratio of 3.2:1, or a combination of both. The starter and finisher diets were the same as the control group. Diets were formulated to meet or exceed all NRC (National Research Council, 1994) nutritional requirements and recommendations in Aviagen's Ross broiler production handbook (Aviagen, 2016). Feed ingredients and calculated nutrient composition in the starter phase are shown in Table 2.1. Grower diets and finisher diets are shown in Table 2.2 and Table 2.3, respectively.

2.2.2 Fatty Acids Determination by GC-MS

The concentrations of long-chain PUFA in feed samples were analyzed using an Agilent Technologies 6890N model gas chromatograph equipped with a 5973N mass spectrometric detector (Agilent Technologies, Wilmington, DE) following the method described by Lima et al. (2017). Feed samples were ground to powder in uniform particle size. Preparation of the fatty acid methyl esters was by the method of direct methylation in Wang et al. (2000). HP-23 capillary column was used for separation. Mass spectrometer was operated in electro impact mode. Helium was used as carrier gas. Each sample was measured in duplicate. Fatty acid composition of the experimental diets in the starter phase, grower phase, and finisher phase are shown in Tables 2.4 and 2.5.

2.2.3 Growth Performance

Animal growth was evaluated by measuring weekly body weights throughout the trial. Feed intake (FI) was calculated by monitoring weekly feed disappearance. Average daily gain (ADG) was calculated as the rate of body weight gain per day. Feed conversion ratio (FCR) was calculated as the ratio of feed intake and body weight gain. At 58 days of age, broilers were harvested in accordance with humane and commercial slaughter procedures. End live weight was taken immediately prior to slaughter. After exsanguination, plucking and evisceration, empty hot carcass weights were taken. Carcasses were then chilled for 1 h in an ice water bath. After the carcasses were sufficiently chilled in a walk-in cold room at 5 °C overnight, chilled carcass weights were recorded, and the left p. major muscle were weighed. P. major weight was calculated as

the two times of the left p. major weight. Right p. major muscles were frozen in a -30°C freezer for further analysis.

2.2.4 Meat Color and Ultimate pH Analysis

Meat color and ultimate pH were evaluated on the left p. major muscle. Meat color was measured using a CR-410 Chroma Meter (Konica Minolta Sensing, Singapore) according to the procedure by Fletcher (1999). The color of each breast fillet was reported in values of L* (lightness), a* (redness), and b* (yellowness). Ultimate pH was evaluated as previously described by Lyon et al. (1985) with the portable pH meter (Model HI98249, Hanna Instruments, Woonsocket, RI). Each sample was measured in triplicate and the average pH value was calculated for each sample. After pH was measured, the p. major muscles were frozen in a -30°C freezer.

2.2.5 Thaw Loss, Cooking Loss, and Tenderness Analysis

Frozen breast muscles were thawed at 4 °C for 24 h. Thawed breast muscles were weighed, and thaw loss was determined by calculating the weight before freezing and after thawing. Thermometers were placed at the center of the breast muscles to monitor their temperature. Muscles were placed in a 150 °C preheated oven and cooked until internal temperature reached 77 °C. Muscles were cooled to room temperature and weighed as a final cooling weight, which was used to calculate cooking loss. Cooking loss was based on the difference between weight after thawing and weight after cooking. The breast muscles were then used for tenderness analysis (Blunt-Meullenet-Owens Razor Shear force) followed by procedure of Lee et al. (2008). Each breast was sheered in five locations by

the blade penetrating the breast perpendicular to the direction of the breast muscle fibers with a TA-XT Texture Analyzer (Texture Technologies Corp, Scarsdale, New York). Shear force and shear energy were recorded to evaluate meat tenderness.

2.2.6 Moisture and Fat Analysis

The right p. major muscles were used to determine percentage of moisture and intramuscular fat content. A total of 210 samples with 10 replicates of 3 samples each were used. The muscle samples were thawed and ground in a food processor until samples were completely homogenous. Each sample was placed in the aluminum pan and weighed. After drying in a 110 °C oven for 48 h, the samples were cooled in a desiccator to room temperature and weighed. Moisture content was calculated based on weight before and after drying. Ether extraction was conducted in AnkomXT 15 extractor (Ankom Technology, Macedon, NY) with filter paper bag holding the dried samples following the instructions of the manufacturer (Ankom Technology, Macedon, NY). The filter paper bag was placed in an oven at 110 °C for 24 h after extraction and was weighed. Fat content was calculated based on weight before and after the ether extraction.

2.2.7 Wooden Breast and White Striping Scores

A total of 210 samples with 10 replicates of 3 samples each were used to evaluate WB and WS scores. Wooden Breast and WS were scored by observation and palpation on the left p. major muscles after the carcass were sufficiently chilled. Wooden Breast scores were based on palpable firmness using a 0 to 3 scale as described by Tijare et al. (2016) with score of 0 meaning not firm and score of 3 meaning the most firm. Score of 1 and 2

are intermediate. White Striping scores were based on white striations as described by Kuttappan et al. (2012b). Score of 0 represents no WS with no white striations and score of 3 represents severe WS with a high level of white striations. Score of 1 and 2 are intermediate.

2.2.8 Statistical Analysis

Data were analyzed as a completely randomized design using PROC MIXED procedure of SAS version 9.4 software (SAS Institute, 2013). Individual pen was identified as the experimental unit. Dietary treatments were used as a fixed effect. Least square means were estimated with the LSMEANS procedure and separated with the PDIFF option. Data from VE supplementation treatments both in starter and grower phases was combined to determine VE effect by setting up orthogonal contrasts. Effect of n-3 fatty acids was determined by setting up orthogonal contrasts using data from n-3 fatty acids supplementation treatments both in starter and grower phases. Data from VE and n-3 fatty acids supplementation treatments both in starter and grower phases was combined to determine combination effect by setting up orthogonal contrasts. Significance was accepted at $P \leq 0.05$.

2.3 Results

2.3.1 Growth Performance

Growth performance of the broilers in this study is shown in Table 2.6. During the starter phase (0 to 10 day), broilers fed with different dietary treatments did not have a

significant difference in FI (P = 0.79) or FCR (P = 0.40). However, broilers fed with increased dietary n-3 fatty acids (n-6/n-3 ratio of 3.2:1) in the starter diets had lower ADG compared with the control group (P = 0.049). Broilers supplemented with increased levels of n-3 fatty acids (n-6/n-3 ratio of 3.2:1) in starter diets had lower ADG than supplemented in grower diets during starter phase (P = 0.016). Additionally, supplemental VE (200 IU/kg) in the grower diets had a decreased FCR (P = 0.035). Neither FI (P = 0.59) nor FCR (P = 0.42) was different among the dietary groups during the finisher phase (25 to 58 day). Nevertheless, broilers in the starter n-3 group had decreased ADG than the control group (P = 0.040) and the grower n-3 group (P = 0.011) during the finisher phase. No significant effect of VE, n-3 fatty acids, or combination of both was shown on broiler growth performance during the entire experiment (P > 0.05). However, there was a trend that increased VE supplementation decreased FCR in grower phase (P = 0.06) while n-3 fatty acids increased FCR in finisher phase (P = 0.09).

2.3.2 Meat Yield

No significant effect of VE, n-3 fatty acids, or the combination of both was shown on broiler meat yield (P > 0.05; Table 2.7). However, n-3 fatty acids supplementation (n-6/n-3 ratio of 3.2:1) in the starter diets significantly decreased final body weight (P = 0.024), hot carcass weight (P = 0.049), and chilled carcass weight (P = 0.024) of broilers compared with the control group. Broilers supplemented with increased levels of both VE (200 IU/kg) and n-3 fatty acid (n-6/n-3 ratio of 3.2:1) in starter diets did not show a

difference in meat yield compared with control group ($P > 0.05$). There was no significant difference in p. major muscle weight among each group ($P = 0.90$).

2.3.3 Meat Color and Ultimate pH

The meat quality data is presented in Table 2.8. Broilers fed a starter diet with both VE (200 IU/kg) and n-3 fatty acids (n-6/n-3 ratio of 3.2:1) had decreased L* (lightness) values compared to the control group ($P = 0.027$), starter VE ($P = 0.050$), and starter n-3 group ($P = 0.045$). Supplemental VE (200 IU/kg) and n-3 fatty acids (n-6/n-3 ratio of 3.2:1) in the grower diets increased b* (yellowness) in the breast muscle compared to supplementing with n-3 fatty acids in the grower diets ($P = 0.039$). There was no significant difference in pH value among the different dietary treatments ($P = 0.60$) and no significant effect of VE, n-3 fatty acids, or combination of both on meat quality ($P > 0.05$).

2.3.4 Thaw Loss, Cooking Loss, and Tenderness

The combination of both VE (200 IU/kg) and n-3 fatty acids (n-6/n-3 ratio of 3.2:1) in the starter diets significantly increased shear force compared with the control group ($P = 0.046$). Breast muscle from the starter VE group had a lower shear force than the grower VE group ($P = 0.049$). No significant difference was shown on thaw loss, cook loss, or shear energy in any group compared with the control group ($P > 0.05$).

2.3.5 Moisture and Fat Content

Results of the proximate analysis are shown in Table 2.9. Breast muscle from the starter VE and n-3 group contained lower moisture content compared with the other groups ($P \leq 0.05$). The breast muscle of broilers supplemented with an increased concentration of

VE (200 IU/kg) in the starter diets or grower diets had lower fat content than the breast muscle from the starter n-3 group ($P = 0.017$). Other than these significant instances, there were no other significant effects of VE, n-3 fatty acids, or combination of both on moisture and fat content ($P > 0.05$).

2.3.6 Wooden Breast and White Striping Scores

Distribution analysis of the WB and WS scores from the different dietary groups is shown in Figure 2.1. In the control group, percentage of WB with none WB was 18.37%, percentage with a mild score of 1 was 48.98%, percentage with a moderate score of 2 was 24.49%, and percentage with a severe score of 3 was 8.16%. Supplemental VE in the starter diets increased percentage of WB with none or a mild score of 0 (48%) and 1 (24%) and thus decreased the severity of WB with a score of 2 (24%) and 3 (4%) (Figure 2.1A). The higher concentration of VE (200 IU/kg) in the starter diets had a greater effect on reducing WB score than in the grower diets. In contrast, n-3 fatty acid (n-6/n-3 ratio of 3.2:1) in the starter or grower diets increased the severity of WB with moderate and severe scores of 2 and 3, respectively. However, combination of VE and n-3 fatty acids in either the starter or grower diets decreased the severity of WB compared with supplementation of n-3 fatty acids alone in the diets.

The percentage of WS was also modified by the diets. There was a higher percentage of non WS (score of 0) and mild WS (score of 1) and a reduction in the percentage of moderate (score of 2) and severe (score of 3) WS (Figure 2.1B) when broilers were fed with the higher concentration of VE (200 IU/kg) in the starter or grower diets.

The starter VE group showed more of an effect on reducing severity of WS compared to the grower VE group. In contrast, n-3 fatty acids increased the severity of WS in both the starter and grower diets.

2.4 Discussion

The present study compared the effects of VE, n-3 fatty acid, and combination of both during the starter phase (0 to 10 day) or grower phase (11 to 24 day) on growth performance, meat yield, meat quality, and severity of WB and WS. Oxidative stress (Mutryn et al., 2015; Abasht et al., 2016) and inflammation (Mudalal et al., 2015; Sihvo et al., 2017) have been found in WB affected breast muscle by histological changes and genomic analysis. Vitamin E and n-3 fatty acid dietary supplementation was selected due to their antioxidant and anti-inflammatory properties, respectively.

In general, VE supplementation did not impact growth performance and meat yield. These findings were in agreement with previous studies in which neither body weight nor meat yield were affected by dietary VE (Bartov and Frigg, 1992; Sakamoto et al., 2006; Kuttappan et al., 2012b; Cheng et al., 2018). This could be due to the fact that the contents of VE in corn-soybean meal basal diets were sufficient to meet the requirement for growth performance and meat production of broilers and would not be changed by increased concentrations of VE supplementation. Omega-3 fatty acids, on the other hand, had a negative influence on growth performance and meat yield especially when the concentration was increased in starter diets, which was consistent with earlier studies

(Ayerza et al., 2002; Azcona et al., 2008; Navidshad, 2009). Similar results were reported by Hulan (1988) that fish oil enriched diets resulted in lower body weight and higher FCR. They suggested that the poorer growth performance was because of lower palatability and higher calcium levels. It was also reported that PUFA reduced *de novo* synthesis resulting in decreased fat deposition (Smink et al., 2010). This finding may explain the reduction in meat yield when n-3 fatty acids were increased in the diet because meat yield is dependent on muscle amount in relation to fat deposit. Several studies using fish oil in the diets, however, did not find adverse effects on body weight or FCR (Lopez-Ferrer et al., 2001; Farhoomand and Checaniazer, 2009). The contrasting results could be related to the concentration of PUFA. Higher PUFA level is more likely to have an adverse effect on growth performance and meat yield. When increased concentrations of VE were combined with n-3 fatty acids, both growth performance and meat yield had no significant change compared with the control group. Voljč et al. (2011) found that VE reduced lipid peroxidation in meat, and therefore reduced the negative effect on meat yield.

Meat quality is closely related with muscle growth and development. Postnatal muscle growth is dependent on satellite cells, which fuse with multinucleated myofibers, donate their nuclei, and increase protein synthesis capabilities (Moss and Leblond, 1971). This results in muscle growth through hypertrophy or the enlargement of existing muscle fibers. Satellite cells are most active the first week posthatch (Halevy et al., 2000; Mozdziak et al., 2002). They are sensitive to nutritional and environmental changes and will have long-term effects on muscle growth and meat quality (Dangott et al., 2000;

Halevy et al., 2001; Velleman et al., 2014). Sufficient nutrients during the first week posthatch are critical for maximal muscle growth (Halevy et al., 2001; Powell et al., 2014) while nutrient restriction during the first week posthatch influences myogenic genes expression and fat deposition in broilers (Velleman et al., 2010; Powell et al., 2014; Velleman et al., 2014). Therefore, early posthatch nutritional strategies can be used to modify satellite cells and influence muscle structure and meat quality.

Meat color is an important indicator of meat quality. There were no significant differences in a* (redness) in the current study. Lu et al. (2014) reported similar results in their VE treatment in broiler chickens. L* (lightness) decreased when increased VE and n-3 fatty acids were added in starter diets, which is similar to the findings of Cheng et al. (2016). Their study attributed the discoloration of meat to lipid peroxidation. Meat tenderness is another important indicator of meat quality, which depends on a number of factors such as collagen, water and lipid content. Shear force, commonly used to show meat tenderness, was lower when VE was supplemented in starter diets than in grower diets in the current study. Meanwhile, fat content in broilers supplemented with VE in starter group was lower than starter n-3 supplementation group. Papah et al. (2017) showed fat content increased when degenerated muscle fibers were replaced by adipose tissue. Broilers supplemented with VE in the starter diets had lower fat content in the current study, suggesting dietary VE supplementation in during the starter phase may have the potential to reduce muscle fiber degeneration.

Wooden Breast and WS were reduced when VE was increased in starter or grower diets of the broilers. The effect of VE on reducing the myopathies could be due to its antioxidant potential. Several studies have indicated that WB affected broilers are under severe oxidative stress (Mutryn et al., 2015; Abasht et al., 2016) and inflammation (Sihvo et al., 2014; Mudalal et al., 2015). Oxidative stress is considered an imbalance between oxidants and antioxidants (Voljč et al., 2011). Oxygen can be reduced to water during respiration. If oxygen cannot be reduced completely, reactive oxygen species (ROS) with hȳgh oxidizing power will be produced. Oxidative stress occurs when the antioxidant system of an individual cannot remove ROS properly (Panda and Cherian, 2014). The presence of oxidative stress in WB condition could be due to insufficient vascularization, as larger diameter myofibers found frequently in heavy weight fast growing broilers restrict the space for capillaries (Velleman and Nestor, 2003).

Oxidative stress could be reduced by antioxidant scavenging free radicals (Miller et al., 1993). Vitamin E is a very powerful antioxidant known to prevent cells and tissues from oxidative damage (Voljč et al., 2011). Among the eight forms of VE, DL-α-tocopherol acetate is the form commonly used in poultry industry and has a high biological efficiency (Panda and Cherian, 2014). The high efficiency comes from incorporation of α-tocopherol directly into cell membranes where oxidation is initiated (Bou et al., 2009). Therefore, myopathies can be reduced by VE through their highly efficient antioxidant effect. Meanwhile, satellite cells have the highest mitotic activity the first week posthatch (Halevy et al., 2000; Mozdziak et al., 2002), which may explain why extra VE

supplementation in starter diets had a better effect on improving tenderness while reducing the severity of WB and WS compared to the grower diets. Additionally, there was no significant effect of VE, n-3 fatty acids, or combination of both on growth, meat yield, and meat quality. But significant differences were found within each dietary treatment in many parameters, indicating the importance of supplementation time.

In contrast, n-3 fatty acids did not show a beneficial effect on reducing the myopathies. Omega-3 fatty acids, including α-linolenic acid (18:3n-3; ALA), eicosapentaenoic acid (20:5n-3; EPA), and docosahexaenoic acid (22:6n-3; DHA), are essential fatty acids. They have been shown to exert anti-inflammatory effects through altering pro-inflammatory cytokines and adhesion molecules (Simopoulos, 2002; Calder, 2003; Rahimi et al., 2011; Yu et al., 2018). However, there is no reported beneficial effect on improving meat quality and reducing severity of WB (Lopez-Ferrer et al., 2001). Thus, combination of VE and n-3 fatty acids in starter phase did not show greater effect on WB reduction than vitamin E supplementation alone or the control group due to the existence of n-3 fatty acids. Nevertheless, the combination of VE and n-3 fatty acids in the current study showed an effect on reducing the severity of WB compared to n-3 fatty acids supplementation alone.

In conclusion, VE supplementation reduced severity of the WB myopathy without sacrificing growth performance and meat yield. Supplemental VE during the starter phase had a better effect on improving meat quality and reducing WB than supplementation during the grower phase. Omega-3 fatty acids supplementation in starter diets, however,

reduced growth performance and meat yield without showing beneficial effect on reducing WB. Combination of VE and n-3 fatty acids decreased the negative impact of n-3 fatty acids on growth performance and meat yield. Future research needs to be focused on determining the effect of VE on muscle structure and gene expression involved in muscle growth, oxidative stress and inflammation.

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Table 2.1 Feed ingredients and calculated nutritional composition of starter diets[1]

Item	Control	Starter VE	Starter n-3	Starter VE and n-3
Ingredients, % as-fed				
Corn	50.85	50.83	50.85	50.83
Soybean meal	33.71	33.71	33.71	33.71
Poultry byproduct meal	7.50	7.50	7.50	7.50
Sodium chloride	0.22	0.22	0.22	0.22
Limestone	1.10	1.10	1.10	1.10
Dicalcium phosphate	0.47	0.47	0.47	0.47
Premix[2](Akey #7)	0.35	0.35	0.35	0.35
$_{L}$-Lys HCl	0.15	0.15	0.15	0.15
$_{DL}$-Met	0.34	0.34	0.34	0.34
$_{L}$-Thr	0.11	0.11	0.11	0.11
$NaHCO_3$	0.10	0.10	0.10	0.10
Selenium	0.10	0.10	0.10	0.10
Amprolium	1.00	1.00	1.00	1.00
Dl-α-tocopherol acetate	0.001	0.020	0.002	0.020
Soy oil	0.11	0.11	1.58	1.58
Corn oil	3.42	3.42	0.13	0.13
Fish oil	-	-	2.29	2.29
Hydrogenated coconut oil	0.47	0.47	-	-
Calculated Nutrients and energy				
AME, kcal/kg	3016	3015	3018	3017
Protein, %	23.73	23.73	23.73	23.73
Calcium, %	0.96	0.96	0.96	0.96
Available phosphorus, %	0.48	0.48	0.48	0.48
Digestible Lys, %	1.28	1.28	1.28	1.28
Digestible Met+Cys, %	0.95	0.95	0.95	0.95

[1]Broilers in control group were fed with diets with standard levels of vitamin E (VE) and omega-3 (n-3) fatty acids during the entire study (0 to 58 day). Supplementation of VE, n-3 fatty acids, or combination of both was performed during the starter phase (0 to 10 day). Dietary treatments during the grower phase (11 to 24 day) were received the same diets as the control group during the starter phase.

[2]The premix provides the following per kg of diet: vitamin A, 1,715,000 IU; vitamin D3, 6,000 IU; vitamin E, 12,600 IU; vitamin B12, 11 µg; folic acid, 1.5 mg; biotin, 150 µg; calcium carbonate, 25 mg; Mn, 60 mg; Zn, 40 mg; I, 0.33 mg; Fe, 80 mg; Cu, 8 mg; Se, 0.15 mg; and ethoxyquin, 150 mg (Provimi North America, Brookville, OH).

Table 2.2 Feed ingredients and calculated nutritional composition of grower diets[1]

Item	Control	Grower VE	Grower n-3	Grower VE and n-3
Ingredients, % as-fed				
Corn	56.28	50.33	50.35	50.33
Soybean meal	28.10	33.71	33.71	33.71
Poultry byproduct meal	7.50	7.50	7.50	7.50
Sodium chloride	0.23	0.22	0.22	0.22
Limestone	1.02	1.10	1.10	1.10
Dicalcium Phosphate	0.29	0.47	0.47	0.47
Premix[2](Akey #7)	0.35	0.35	0.35	0.35
$_{L}$-Lys HCl	0.15	0.15	0.15	0.15
$_{DL}$-Met	0.30	0.34	0.34	0.34
$_{L}$-Thr	0.09	0.11	0.11	0.11
$NaHCO_3$	0.10	0.10	0.10	0.10
Selenium	0.10	0.10	0.10	0.10
Amprolium	1.00	1.00	1.00	1.00
Dl-α-tocopherol acetate	0.001	0.020	0.001	0.021
Soy oil	0.17	0.17	1.87	1.87
Corn oil	3.81	3.81	0.11	0.11
Fish oil	-	-	2.53	2.53
Hydrogenated coconut Oil	0.52	0.52	-	-
Calculated Nutrients and energy				
AME, kcal/kg	3107	3106	3110	3109
Protein, %	21.56	21.56	21.56	21.56
Calcium, %	0.87	0.87	0.87	0.87
Available phosphorus, %	0.43	0.43	0.43	0.43
Digestible Lys, %	1.15	1.15	1.15	1.15
Digestible Met+Cys, %	0.87	0.87	0.87	0.87

[1]Broilers in control group were fed with diets with standard levels of vitamin E (VE) and omega-3 (n-3) fatty acids during the entire study (0 to 58 day). Supplementation of VE, n-3 fatty acids, or combination of both was performed during the grower phase (11 to 24 day). Dietary treatments during the starter phase (0 to 10 day) were received the same diets as the control group during the grower phase.

[2]The premix provides the following per kg of diet: vitamin A, 1,715,000 IU; vitamin D3, 6,000 IU; vitamin E, 12,600 IU; vitamin B12, 11 µg; folic acid, 1.5 mg; biotin, 150 µg; calcium carbonate, 25 mg; Mn, 60 mg; Zn, 40 mg; I, 0.33 mg; Fe, 80 mg; Cu, 8 mg; Se, 0.15 mg; and ethoxyquin, 150 mg (Provimi North America, Brookville, OH).

Table 2.3 Feed ingredients and calculated nutritional composition of finisher diets[1]

Item	Finisher 1 diets (25 to 38 day)	Finisher 2 diets (39 to 51 day)	Finisher 3 diets (52 to 58 day)
Ingredients, % as-fed			
Corn	61.23	64.23	66.68
Soybean meal	22.98	20.35	18.35
Poultry byproduct meal	7.50	7.50	7.50
Sodium chloride	0.23	0.23	0.23
Limestone	0.94	0.91	0.88
Dicalcium Phosphate	0.10	0.02	-
Premix[2](Akey #7)	0.35	0.35	0.35
$_{L}$-Lys HCl	0.13	0.11	0.12
$_{DL}$-Met	0.27	0.23	0.22
$_{L}$-Thr	0.07	0.06	0.05
$NaHCO_3$	0.10	0.10	0.10
Selenium	0.10	0.10	0.10
Amprolium	1.00	1.00	1.00
Corn oil	3.00	2.71	2.40
Blended Fat	2.00	2.10	2.02
Calculated Nutrients and energy			
AME, kcal/kg	3203	3225	3226
Protein, %	19.58	18.60	17.87
Calcium, %	0.78	0.74	0.72
Available phosphorus, %	0.39	0.37	0.36
Digestible Lys, %	1.02	0.95	0.91
Digestible Met+Cys, %	0.80	0.74	0.71

[1]Broilers in all experimental groups were fed with same finisher diets with standard levels of vitamin E (VE) and omega-3 (n-3) fatty acids.
[2]The premix provides the following per kg of diet: vitamin A, 1,715,000 IU; vitamin D3, 6,000 IU; vitamin E, 12,600 IU; vitamin B12, 11 µg; folic acid, 1.5 mg; biotin, 150 µg; calcium carbonate, 25 mg; Mn, 60 mg; Zn, 40 mg; I, 0.33 mg; Fe, 80 mg; Cu, 8 mg; Se, 0.15 mg; and ethoxyquin, 150 mg (Provimi North America, Brookville, OH).

Table 2.4 Analyzed fatty acid composition of starter and grower diets[1]

Item, %	Starter diets				Grower diets			
	Control	VE	n-3	VE and n-3	Control	VE	n-3	VE and n-3
$C_{8:0}$	0.18	0.00	0.00	0.00	0.00	0.28	0.00	0.00
$C_{10:0}$	0.28	0.20	0.00	0.00	0.20	0.24	0.00	0.00
$C_{12:0}$	2.14	1.56	0.00	0.00	1.59	1.84	0.00	0.00
$C_{14:0}$	0.99	0.74	1.57	1.83	0.62	0.82	1.74	1.80
$C_{15:0}$	0.00	0.00	0.13	0.25	0.00	0.00	0.15	0.21
$C_{16:0}$	14.18	11.48	13.26	14.74	11.34	11.50	14.76	14.44
$C_{16:1}$	0.45	0.39	2.58	2.84	0.31	0.37	0.15	2.72
$C_{17:0}$	0.00	0.00	0.20	0.36	0.00	0.05	0.32	0.29
$C_{17:1}$	0.00	0.00	0.00	0.19	0.00	0.00	0.00	0.00
$C_{18:0}$	2.49	2.59	3.95	3.16	2.89	2.91	3.60	3.86
$C_{18:1}$	29.44	26.14	20.20	19.78	26.16	25.08	21.13	20.75
$C_{18:2}$ (n-6)	46.18	53.79	41.37	41.53	54.02	54.09	43.91	40.96
$C_{18:3}$ (n-6)	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00
$C_{18:3}$ (n-3)	2.26	1.82	3.67	3.64	1.79	1.77	3.49	3.40
$C_{20:0}$	0.45	0.40	0.40	0.34	0.37	0.37	0.32	0.36
$C_{20:1}$	0.29	0.27	0.47	0.39	0.24	0.23	0.40	0.40
$C_{20:2}$	0.00	0.00	0.08	0.16	0.00	0.04	0.15	0.13
$C_{20:4}$ (n-6)	0.17	0.17	0.48	0.43	0.13	0.06	0.40	0.41
$C_{20:3}$ (n-3)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
$C_{20:5}$ (n-3)	0.00	0.00	4.37	4.05	0.00	0.00	3.81	4.00
$C_{22:0}$	0.26	0.20	0.38	0.31	0.17	0.14	0.22	0.34
$C_{22:5}$ (n-3)	0.00	0.00	0.90	0.78	0.00	0.00	0.66	0.74
$C_{22:6}$ (n-3)	0.00	0.00	5.63	4.93	0.00	0.00	4.49	4.91
$C_{24:0}$	0.24	0.25	0.37	0.29	0.17	0.22	0.25	0.29
SFA[2]	21.20	17.42	20.26	21.29	17.34	18.36	21.36	21.58
MUFA[3]	29.73	26.41	20.67	20.37	26.41	25.31	21.53	21.15
PUFA[4]	48.61	55.78	56.50	55.51	55.93	55.96	56.96	54.55
n-3	2.26	1.82	14.57	13.39	1.79	1.77	12.47	13.06
n-6	46.35	53.96	41.85	41.96	54.15	54.15	44.34	41.37
n-6:n-3	20.5	29.6	2.9	3.1	30.3	30.6	3.6	3.2

[1]Broilers in control group were fed with diets with standard levels of vitamin E (VE) and omega-3 (n-3) fatty acids during the entire study (0 to 58 day). Supplementation of VE, n-3 fatty acids, or combination of both was performed during the starter phase (0 to 10 day) or grower phase (11 to 24 day).
[2]SFA = Saturated fatty acids.
[3]MUFA = Monounsaturated fatty acids.
[4]PUFA = Polyunsaturated fatty acids.

Table 2.5 Analyzed fatty acid composition of finisher diets[1]

	Finisher 1 diets (25 to 38 day)	Finisher 2 diets (39 to 51 day)	Finisher 3 diets (52 to 58 day)
$C_{8:0}$	0.00	0.00	0.00
$C_{10:0}$	0.95	0.00	0.00
$C_{12:0}$	0.00	0.00	0.00
$C_{14:0}$	0.17	0.13	0.14
$C_{15:0}$	0.00	0.01	0.00
$C_{16:0}$	12.96	13.81	11.19
$C_{16:1}$	0.68	0.38	0.58
$C_{17:0}$	0.00	0.00	0.00
$C_{17:1}$	0.00	0.00	0.00
$C_{18:0}$	3.24	2.03	1.75
$C_{18:1}$	28.37	18.09	25.20
$C_{18:2}$ (n-6)	50.28	62.13	47.56
$C_{18:3}$ (n-6)	0.00	0.00	10.54
$C_{18:3}$ (n-3)	1.96	2.15	1.87
$C_{20:0}$	0.36	0.36	0.31
$C_{20:1}$	0.28	0.19	0.25
$C_{20:2}$	0.13	0.11	0.00
$C_{20:4}$ (n-6)	0.24	0.23	0.22
$C_{20:3}$ (n-3)	0.00	0.00	0.00
$C_{20:5}$ (n-3)	0.00	0.00	0.00
$C_{22:0}$	0.18	0.18	0.18
$C_{22:5}$ (n-3)	0.00	0.00	0.00
$C_{22:6}$ (n-3)	0.00	0.00	0.00
$C_{24:0}$	0.19	0.20	0.20
SFA[2]	18.07	16.72	13.77
MUFA[3]	28.66	18.28	25.46
PUFA[4]	52.60	64.62	60.19
n-3	1.96	2.15	1.87
n-6	50.51	62.36	58.32
n-6:n-3	25.8	29.0	31.2

[1]Broilers in control group were fed with diets with standard levels of vitamin E (VE) and omega-3 (n-3) fatty acids during the entire study (0 to 58 day). Supplementation of VE, n-3 fatty acids, or combination of both was performed during the starter phase (0 to 10 day) or grower phase (11 to 24 day).
[2]SFA = Saturated fatty acids; [3]MUFA = Monounsaturated fatty acids; [4]PUFA = Polyunsaturated fatty acids.

Table 2.6 Effect of vitamin E and omega-3 fatty acids on growth performance of broilers[1]

Item	Treatments							SEM[2]	*P*-value		
	Control	Starter VE	Starter n-3	Starter VE and n-3	Grower VE	Grower n-3	Grower VE and n-3		VE	n-3	VE and n-3
Starter phase (0 to 10 day)											
ADG[3]	15.71^{abc}	16.42^{a}	14.03^{d}	14.61^{abcd}	14.72^{abcd}	16.41^{ab}	14.64^{abcd}	0.20	0.84	0.49	0.14
FI[4]	276.87	268.39	274.79	277.79	271.96	275.85	242.18	3.85	0.69	0.93	0.33
FCR[5]	0.99	0.92	1.14	1.09	1.06	0.97	0.96	0.02	0.99	0.45	0.73
Grower phase (10 to 24 day)											
ADG	38.65	39.38	36.68	39.78	39.29	38.37	36.61	0.50	0.65	0.46	0.77
FI	1019.96^{ab}	1019.57^{ab}	934.24^{ab}	1064.80^{a}	899.84^{b}	1009.47^{ab}	968.65^{ab}	10.74	0.25	0.36	0.95
FCR	1.82^{ab}	1.70^{abc}	1.73^{abc}	1.86^{a}	1.58^{c}	1.77^{abc}	1.81^{abc}	0.03	0.06	0.48	0.88
Finisher phase (24 to 58 day)											
ADG	80.73^{bc}	76.59^{a}	75.34^{d}	82.69^{bcd}	78.29^{bcd}	83.12^{b}	80.26^{bcd}	0.77	0.13	0.49	0.74
FI	4199.81	4081.89	4221.88	4541.95	4261.92	4489.25	4263.25	38.22	0.87	0.39	0.29
FCR	1.52	1.52	1.62	1.60	1.59	1.58	1.53	0.02	0.42	0.09	0.33

[a-d]Values within a row without a common letter are significantly different ($P \leq 0.05$).
[1]Values are means of 10 pens per treatment, each pen included three birds. Broilers in control group were fed with diets with standard levels of vitamin E (VE) and omega-3 (n-3) fatty acids during the entire study (0 to 58 day). Supplementation of extra VE, n-3 fatty acids, or combination of both was performed during the starter phase (0 to 10 day) or grower phase (11 to 24 day).
[2]SEM=Standard error of means.
[3]ADG=Average daily gain (g).
[4]FI=Feed intake (g).
[5]FCR=Feed conversion ratio.

Table 2.7 Effect of vitamin E and omega-3 fatty acids on meat yield of broilers[1]

Item	Treatments							SEM[2]	*P*-value		
	Control	Starter VE	Starter n-3	Starter VE and n-3	Grower VE	Grower n-3	Grower VE and n-3		VE	n-3	VE and n-3
Final body weight	3593.56[a]	3466.45[ab]	3344.91[b]	3660.58[a]	3484.78[ab]	3571.41[ab]	3521.81[ab]	33.00	0.20	0.14	0.98
Hot carcass weight	2707.99[a]	2602.52[ab]	2537.79[b]	2800.40[a]	2650.02[ab]	2694.61[ab]	2663.03[ab]	26.42	0.26	0.21	0.75
Chilled carcass weight	2792.27[a]	2691.53[ab]	2585.96[b]	2888.32[a]	2727.37[ab]	2799.75[a]	2739.22[ab]	27.60	0.28	0.20	0.78
P. major weight[3]	662.46	641.77	649.23	689.32	665.32	672.58	659.53	8.76	0.72	0.95	0.64

[a, b]Values within a row without a common letter are significantly different ($P \leq 0.05$).
[1]Values are means of 10 pens per treatment, each pen included three birds. Meat yield was measured at the end of the study. Broilers in control group were fed with diets with standard levels of vitamin E (VE) and omega-3 (n-3) fatty acids during the entire study (0 to 58 day). Supplementation of extra VE, n-3 fatty acids, or combination of both was performed during the starter phase (0 to 10 day) or grower phase (11 to 24 day).
[2]SEM=Standard error of means.
[3]Pectoralis major (p. major) weight was calculated as the two times of the left p. major weight.

Table 2.8 Effect of vitamin E and omega-3 fatty acids on meat quality of broilers[1]

Item	Treatments							SEM[2]	*P*-value		
	Control	Starter VE	Starter n-3	Starter VE and n-3	Grower VE	Grower n-3	Grower VE and n-3		VE	n-3	VE and n-3
l*	64.11[a]	64.07[a]	64.10[a]	62.61[b]	63.75[ab]	63.89[ab]	63.94[ab]	0.18	0.70	0.83	0.11
a*	12.93	12.53	12.96	13.22	12.88	12.85	12.55	0.10	0.44	0.92	0.87
b*	12.89[ab]	12.38[b]	13.35[ab]	12.81[ab]	12.91[ab]	12.49[b]	13.90[a]	0.16	0.62	0.95	0.35
pH	5.79	5.79	5.82	5.79	5.79	5.80	5.82	0.01	0.99	0.22	0.52
Thaw loss (%)	1.78	1.61	1.45	1.47	1.61	1.54	1.68	0.08	0.44	0.20	0.38
Cook loss (%)	19.59	19.90	18.76	19.21	20.41	19.08	19.95	0.36	0.57	0.51	0.99
Shear force (N)	14.74[b]	14.70[b]	14.86[ab]	15.95[a]	15.78[a]	14.16[b]	14.88[ab]	0.17	0.29	0.64	0.17
Shear energy (N/mm)	1.87	1.92	1.86	1.97	1.96	1.83	1.85	2.05	0.17	0.74	0.42

[a-c]Values within a row without a common letter are significantly different ($P \leq 0.05$).
[1]Values are means of 10 pens per treatment, each pen included three birds. Broilers in control group were fed with diets with standard levels of vitamin E (VE) and omega-3 (n-3) fatty acids during the entire study (0 to 58 day). Supplementation of extra VE, n-3 fatty acids, or combination of both was performed during the starter phase (0 to 10 day) or grower phase (11 to 24 day).
[2]SEM=Standard error of means.

Table 2.9 Effect of vitamin E and omega-3 fatty acids on moisture and fat contents of breast muscle[1]

Item (%)	Treatments							SEM[2]	*P*-value		
	Control	Starter VE	Starter n-3	Starter VE and n-3	Grower VE	Grower n-3	Grower VE and n-3		VE	n-3	VE and n-3
Moisture	75.75[a]	75.61[a]	75.69[a]	75.10[b]	75.69[a]	75.63[a]	75.93[a]	0.06	0.56	0.61	0.18
Fat	1.39[ab]	1.30[b]	1.50[a]	1.47[ab]	1.34[b]	1.43[ab]	1.43[ab]	0.02	0.25	0.23	0.40

[a, b]Values within a row without a common letter are significantly different ($P \leq 0.05$).
[1]Values are means of 10 pens per treatment, each pen included three birds. Broilers in control group were fed with diets with standard levels of vitamin E (VE) and omega-3 (n-3) fatty acids during the entire study (0 to 58 day). Supplementation of extra VE, n-3 fatty acids, or combination of both was performed during the starter phase (0 to 10 day) or grower phase (11 to 24 day).
[2]SEM=Standard error of means.

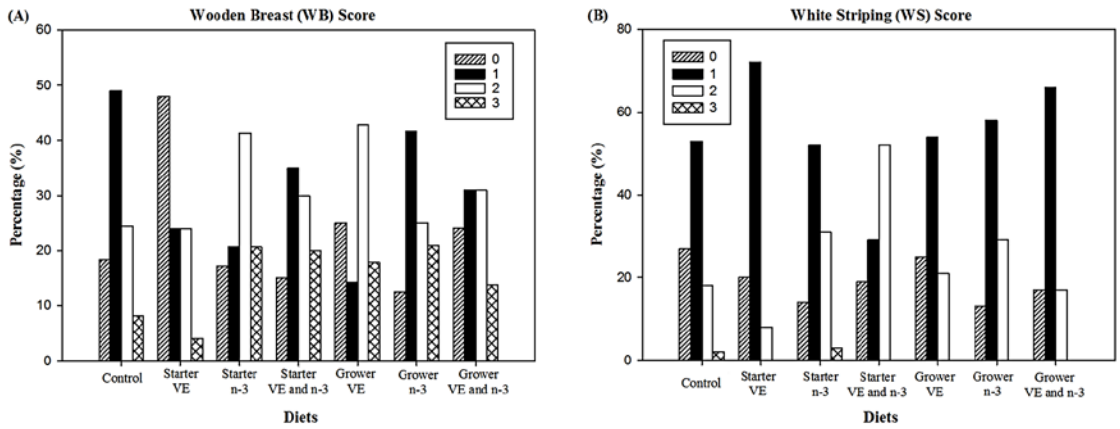

Figure 2.1 Effect of vitamin E (VE) and omega-3 (n-3) fatty acids on Wooden Breast (WB) score (A) and White Striping (WS) score (B) of the breast muscle. A total of 210 breast muscles with 10 replicates of 3 muscles each were evaluated. Broilers in the control group were fed with corn-soybean meal basal diet with VE (10 IU /kg) and n-3 fatty acids (n-6/n-3 ratio of 30.2:1) at a standard level during the entire study (0 to 58 day). Starter VE, starter n-3, starter VE and n-3 were supplemented with 200 IU/kg of VE (200 IU/kg), n-3 fatty acids (n-6/n-3 ratio of 3.2:1), or a combination of both during the starter phase (0 to 10 day). Grower VE, grower n-3, grower VE and n-3 were supplemented with 200 IU/kg of VE (200 IU/kg), n-3 fatty acids (n-6/n-3 ratio of 3.2:1), or a combination of both during the grower phase (11 to 24 day). Scores of WB and WS were based on palpation and visual observation. Score 0 = none, 1 = mild, 2 = moderate, 3 = severe.

Chapter 3: Effect of Early Posthatch Supplementation of Vitamin E and Omega-3 Fatty Acids on the Severity of Wooden Breast, Breast Muscle Morphological Structure, and Gene Expression in the Broiler Breast Muscle *

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Abstract

Wooden Breast (WB) has arisen primarily in the breast muscle of commercial broilers. It is characterized by palpation of a rigid pectoralis major (p. major) muscle and is under severe oxidative stress and inflammation. Previous studies have shown that vitamin E (VE) has antioxidant properties and omega-3 (n-3) fatty acids have an anti-inflammatory effect. The objectives of this study were to identify the effects of VE and n-3 fatty acids on the severity of WB, morphological structure of the p. major muscle, expression of genes likely associated with WB, and to determine the most beneficial supplementation period. A total of 210 Ross 708 broilers were randomly assigned into seven treatments with 10 replicates of three birds each. The control group received a corn-soybean meal basal diet during the entire study (0 to 58 day). Supplementation of VE (200

IU/kg), n-3 fatty acids (n-6/n-3 ratio of 3.2:1), or combination of both were fed during the starter phase (0 to 10 day) or grower phase (11 to 24 day). All broilers were harvested at 58 day of age. Morphological assessment of the p. major muscle included myofiber width, perimysial and endomysial connective tissue space, overall morphological structure, and scoring of WB microscopically. Gene expression was measured using Nanostring analysis. Genes associated with muscle development and growth factors, inflammation, extracellular matrix, and glucose metabolism were differentially expressed in the p. major muscle of the broilers supplemented with VE in the grower diet. More than two times giant myofibers (≥ 70 μm) were found in the group supplemented with VE and n-3 fatty acids in the starter diet compared to the group fed VE in the grower diet (P = 0.02). Microscopic evaluation showed that VE supplementation in the grower diet had a 16.19% increase of muscle with no WB compared to the control group (P = 0.05). These data suggest that supplementation of VE during the grower phase may reduce the severity of WB in broilers.

3.1 Introduction

The demand for poultry meat among consumers has increased notably due to its lower cost, high protein, and convenience in cooking (Bordoni and Danesi, 2017; Mottet and Tempio, 2017). To meet consumer demand, broilers are continuously selected for rapid growth, improved feed conversion, and increased breast meat yield (Brewer et al., 2012; Chatterjee et al., 2019). Poultry meat production in 2019 is more than five times of that in 1970 (National Chicken Council, 2020). However, selection for rapid growth including the

breast muscle has resulted in meat quality defects (Dransfield and Sosnicki, 1999; Petracci et al., 2013, 2015; Tijare et al., 2016). One of the primary myopathies, Wooden Breast (WB), has emerged within the broiler industry worldwide (Sihvo et al., 2014; Kuttappan et al., 2016; Tasoniero et al., 2016). Wooden Breast is phenotypically characterized by a hard breast muscle upon palpation (Sihvo et al., 2014). It is estimated that around 85% of the commercial broilers are at least mildly affected by the WB myopathy (Kuttappan et al., 2017). This myopathy has created considerable economic losses of over $200 million dollars per year due to the hardness of the breast muscle, lack of palatability, and product downgrades (Sihvo et al., 2014; Kuttappan et al., 2016).

Histologically, WB has moderate or severe myodegeneration along with different levels of myofiber necrosis (Papah et al., 2017), fibrosis (Sihvo et al., 2014; Velleman and Clark, 2015), and inflammatory cell accumulation (Sihvo et al., 2014, 2017). Gene expression analysis and metabolomics analysis have found that genes altered in WB muscle tissue are closely related with muscle development (Velleman and Clark, 2015; Abasht et al., 2016; Zambonelli et al., 2017), hypoxia and oxidative stress (Mutryn et al., 2015; Abasht et al., 2016; Brothers et al., 2019), response to inflammation (Mutryn et al., 2015; Zambonelli et al., 2017), calcium signaling (Mutryn et al., 2015; Zambonelli et al., 2017), and dysregulation of lipid and glucose metabolism (Abasht et al., 2016; Brothers et al., 2019). These findings are strongly suggestive that the WB myopathy is associated with oxidative stress and inflammation. Therefore, the severity of WB can be potentially

decreased when oxidative stress and inflammation are reduced to levels that are not harmful to the breast muscle.

Oxidative stress is defined as an imbalance between oxidants and antioxidants in cells and tissues (Voljč et al., 2011). Reactive oxygen species (ROS) are synthesized when oxygen is not reduced completely. Oxidative stress results when the antioxidant system is not able to remove ROS properly (Panda and Cherian, 2014). Vitamin E (VE) is an antioxidant that removes free radicals and prevents oxidative stress (Kuttappan et al., 2012; Niki, 2016). DL-α-tocopherol acetate is the commonly used form of VE in the poultry industry and has high biological efficacy (Hosomi et al., 1997; Panda and Cherian, 2014). It can remove free radical intermediates by reacting with lipid radicals from the lipid peroxidation chain reaction (Niki et al., 1993). This thereby terminates the propagation reaction from continuing and prevents cell membrane oxidation (Niki et al., 1991). Chapter 2 showed that supplementation of VE (200 IU/kg) in the starter diet (0 to 10 day) or grower diet (11 to 24 day) increased the percentage of birds without WB affected breast muscle compared to the control diet at 58 days of age. Additionally, omega-3 (n-3) polyunsaturated fatty acids can reduce inflammation and enhance muscle function (Calder, 2006; Ewaschuk et al., 2014; Yu et al., 2018). Synergistic effects of VE and n-3 fatty acids have been shown to reduce meat oxidation and inflammation (Taulescu et al., 2011; EI-Samee et al., 2019). Thus, reducing oxidative stress and inflammation in broilers through VE and n-3 fatty acids administration will likely have beneficial implications on the structure of the pectoralis

major (p. major) muscle, severity of the WB myopathy, and expression of genes related with muscle development, oxidative stress, and inflammation.

Although previous studies have identified that VE is an antioxidant (Kuttappan et al., 2012; Niki, 2016) and n-3 fatty acids have anti-inflammatory effects (Calder, 2006; Ewaschuk et al., 2014; Yu et al., 2018), there are no published studies showing the effects of VE and n-3 fatty acids on the morphological structure of the p. major muscle, and expression of genes likely associated with WB. Therefore, the objectives of this study were to identify the effects of dietary VE and n-3 fatty acids independently or in combination when supplemented during the starter phase (0 to 10 day) or grower phase (11 to 24 day) on the severity of the WB myopathy. Assessment of the morphological structure of the p. major muscle, and expression of genes related with muscle formation and growth, growth factors, hypoxia, oxidative stress and inflammation, extracellular matrix, cell structure and migration, calcium regulation, and glucose metabolism in the p. major muscle were measured to determine the most beneficial dietary supplementation period to mitigate WB development in broilers.

3.2 Materials and Methods

3.2.1 Birds and Experimental Diets

All bird activities were approved by the Institutional Animal Care and Use Committee of The Ohio State University. A total of 210 commercial Ross 708 broiler chicks were individually wing banded and placed into pens immediately after hatch.

Broilers had ad libitum access to feed and water. Birds were assigned to seven experimental groups in a completely randomized design (Figure 3.1). There were 10 pens per treatment, and each pen included three birds. The control group was fed a corn-soybean meal basal diet with VE (DL-α-tocopherol acetate, 10 IU /kg) and n-3 fatty acids (n-6/n-3 ratio of 30.2:1) at a standard level during the starter (0 to 10 day), grower (11 to 24 day), and finisher phases (25 to 58 day). Additional supplemental VE or n-3 fatty acids were fed during the starter or grower phases. For the starter dietary supplementation, starter VE, starter n-3, and starter VE and n-3 groups were fed the basal starter diet supplemented at concentrations of 200 IU/kg diet of VE, n-3 fatty acids with a n-6/n-3 ratio of 3.2:1, or a combination of both. The grower and finisher diets were the same as the control group. For the grower dietary supplementation, grower VE, grower n-3, and grower VE and n-3 groups were fed the basal grower diets supplemented at concentrations of 200 IU/kg diet of VE, n-3 fatty acids with a n-6/n-3 ratio of 3.2:1, or a combination of both. The starter and finisher diets were the same as the control group. Diets were formulated to meet or exceed all NRC (National Research Council, 1994) nutritional requirements and recommendations in Aviagen's Ross broiler production handbook (Aviagen, 2016). Feed ingredients and nutrient composition have been reported in Chapter 2. At 58 days of age, all broilers were harvested in accordance with humane and commercial slaughter procedures. Samples of p. major muscle were obtained from each broiler for evaluation of p. major muscle morphology and gene expression.

3.2.2 Pectoralis Major Muscle Morphology

Samples for muscle histology were collected according to Velleman et al. (2003b). In brief, after the skin was removed, muscle fibers in the anterior portion of the muscle were dissected following the fiber orientation and tied to wooden applicator sticks to prevent contraction. Tissue samples were fixed in 10% (vol/vol) buffered formalin (pH 7.0) and stored at 4 °C. Histological samples were processed with dehydration in a graded series of alcohol and cleared using Pro-Par Clearant (Anatech, Battle Creek, MI), and paraffin embedded based on the method of Jarrold et al. (1999). Samples were then cross sectioned at a 5 µm thickness, and mounted on Starfrost Adhesive slides (Mercedes Medical, Sarasota, FL). Each slide contained four sections. The sections were stained with hematoxylin and eosin and imaged with a QImaging digital camera (QImaging, Burnaby, BC, Canada) attached to an Olympus IX 70 microscope (Olympus America, Mellville, NY).

Four photomicrographs from each sample were taken for p. major muscle morphology score and WB myopathy score evaluation. The p. major muscle morphology score was evaluated as described by Velleman et al. (2003b). Samples were scored using a one to five scale by four trained panelists. A score of one was given to samples with limited or no perimysial or endomysial connective tissue spacing, and excessive myofiber degradation and necrosis. A score of five was given to samples with well-structured muscle fiber bundles and myofibers with ample perimysial and endomysial connective tissue spacing. Scores of two to four were intermediate. Wooden Breast myopathy scores were

recorded based on the degree of fibrosis, necrosis, and immune cell infiltration, with a score of zero representing no necrosis, fibrosis, or immune cell infiltration, a score of one representing minimal necrosis, fibrosis, and immune cell infiltration, a score of two being intermediate, and a score of three representing severe necrosis, fibrosis, and immune cell infiltration.

Myofiber diameter, perimysial and endomysial width were measured from four photomicrographs. At least 20 measurements were taken in each photomicrograph using Image Pro software (Media Cybernectics, Bethesda, MD). Myofiber widths were grouped into the following categories based on Clark et al. (2017): small (fiber width < 20 μm), intermediate (20 μm ≤ fiber width < 40 μm), large (40 μm ≤ fiber width < 70 μm), and giant fibers (fiber width ≥ 70 μm).

3.2.3 Nanostring nCounter Gene Expression Analysis

About 0.50 g of p. major muscle samples were collected and maintained in RNAlater (Ambion, Grand Island, NY). After 24 h, RNAlater was removed and the samples were stored at -20°C until RNA extraction. Total RNA was extracted from p. major muscle samples using RNAzol RT (Molecular Research Center, Cincinnati, OH) according to manufacturer's protocol. Gene expression analysis was completed by Nanostring nCounter Analysis (Nanostring Technologies, Seattle, WA) following the procedure described in Geiss et al. (2008). Genes whose expression is associated with muscle formation and growth, growth factors, hypoxia, oxidative stress and inflammation, extracellular matrix, cell structure and migration, calcium regulation, and glucose

metabolism were selected as target sequences to be measured (Table 3.1). Codesets containing reporter and capture probes for target sequences were designed by Nanostring (Nanostring Technologies, Seattle, WA). The RNA samples were hybridized to the codsets, incubated for 16 h, and digitally analyzed for quantification.

3.2.4 Statistical Analysis

Data of fiber width, perimysial and endomysial width, and morphology score were analyzed as a completely randomized design using PROC MIXED procedure of SAS version 9.4 software (SAS Institute INC., Cary, NC). Wooden Breast score was analyzed with PROC GENMOD procedure of SAS. Individual pen was identified as the experimental unit. Dietary treatments were used as a fixed effect. Least square means were estimated with the LSMEANS procedure and separated with the PDIFF option. Significance was accepted at $P \leq 0.05$. Gene expression presented as fold changes were analyzed with the Nanostring nSolver version 4.0 software (Nanostring Technologies, Seattle, WA). Fold change for each gene was calculated as the ratio between each dietary treatment and the control group. If ratio was higher than one, fold change was equal to the ratio. If ratio was lower than one, fold change was the negative inverse of the ratio. Fold differences of gene expression among the dietary treatments were calculated with the fold changes. If one of the fold change was positive and another was negative, the fold difference of gene expression between the two treatments was calculated as the percentage of the multiplication of their fold changes subtract 100%. If the fold changes were both positive or negative, the fold difference of gene expression between the two treatments was

calculated as the percentage of the division of their fold changes subtract 100%. Heatmap was generated by RStudio version 3.5.2 with the R pheatmap package (RStudio INC., Boston, MA).

3.3 Results

3.3.1 Pectoralis Major Muscle Myofiber Width, and Perimysial and Endomysial Space

Pectoralis major muscle myofiber width from all treatments is shown in Table 3.2. The average myofiber width and percentage of intermediate myofiber width (20 μm ≤ fiber width < 40 μm) were not significantly different among the dietary treatments ($P > 0.05$). However, there was a trend that broilers supplemented with n-3 fatty acids during the grower phase (11 to 24 day) had a 42% increase of small myofibers (fiber width < 20 μm) compared to VE supplemented broilers in the starter phase (0 to 10 day; $P = 0.09$). Broilers fed dietary VE (200 IU/kg) in the starter phase had 1.14 times of large myofibers (40 μm ≤ fiber width < 70 μm) than broilers fed dietary n-3 fatty acids (n-6/n-3 ratio of 3.2:1) in the grower phase ($P = 0.03$). Meanwhile, 2.10 times of giant myofibers with widths greater than 70 μm were identified in the group supplemented with VE and n-3 fatty acids in the starter phase compared to the group supplemented with VE in the grower phase ($P = 0.02$). There was no significant difference of the dietary treatments in perimysial or endomysial connective tissue space in any of the treatment groups as shown in Table 3.3 ($P > 0.05$).

3.3.2 Morphology Score and Wooden Breast Score

Figure 3.2 shows representative photomicrographs of the p. major muscle histology with morphology scores of one to five. Figure 3.2A shows samples with a score of 1 with indistinct myofibers and limited or no perimysial or endomysial connective tissue space. Figure 3.2B shows samples with a score of 2 with excessive regenerating and hypertrohic fibers, and limited perimysial or endomysial connective tissue space. Figure 3.2C shows samples with a score of 3 with intermediate regenerating fibers, and perimysial or endomysial connective tissue space. Figure 3.2D shows samples with a score of 4 with minimal regenerating fibers, and ample perimysial and endomysial connective tissue space. Figure 3.2E shows samples with a score of 5 with distinct myofibers, and ample perimysial and endomysial connective tissue space. Vitamin E supplementation during the grower phase significantly increased morphology scores by 16% compared to the group supplemented with n-3 fatty acids in the grower phase ($P = 0.04$; Table 3.3).

Representative images of WB scores of zero to three are shown in Figure 3.3. Figure 3.3A shows samples with a score of 0 with no necrosis, fibrosis, or immune cell infiltration, indicating no WB myopathy in the p. major muscle. Figure 3.3B shows samples with a score of 1 with minimal necrosis, fibrosis, and immune cell infiltration, indicating mild WB myopathy. Figure 3.3C shows samples with a score of 2 shows samples with intermediate necrosis, fibrosis, and immune cell infiltration, indicating moderate WB myopathy. Figure 3.3D shows samples with a score of 3 with extensive fibrosis, necrosis,

and immune cell infiltration, indicating severe WB myopathy. Figure 3.4 shows extensive mononucleated inflammatory cell infiltration associated with WB myopathy.

Table 3.4 shows the WB score of broiler p. major muscle in the dietary treatments. The percentage of WB score zero was 16.19% increased in the broilers supplemented with VE during the grower phase compared to the control group ($P = 0.05$). Supplementation of n-3 fatty acids in the grower diet increased the percentage of WB score one compared to the group supplemented with n-3 fatty acids in the starter diet ($P = 0.03$) and VE in the grower diet ($P = 0.01$). When the broilers were fed dietary n-3 fatty acids during the starter phase, percentage of WB score two was increased compared to the control group ($P = 0.04$). Supplementation of n-3 fatty acids in the starter diet decreased the percentage of WB score three compared to supplementation of VE in the grower diet ($P = 0.02$).

3.3.3 Nanostring nCounter Gene Expression

Figure 3.5 depicts the gene expression heatmap of p. major muscle of the broilers supplemented with different dietary treatments. The data reveals differential gene expression among treatments. Normalized gene expression abundance is color-coded according to the legend. Redness represents up-regulation of genes while blueness represents down-regulation of genes compared to the control group. Gene fold changes are in Table 3.5. In terms of muscle formation and growth, supplemental VE in the grower diet had a 14% increase of expression of myogenic differentiation factor 1 (*MYOD1*; $P = 0.04$) compared to VE and n-3 fatty acids supplementation in the starter diet. Expression of transforming growth factor beta 1 (*TGFB1*) in the broilers supplemented with VE and n-3

fatty acids during the starter phase was 1.40 times of expression in VE supplemented broilers during the grower phase ($P = 0.02$). With regard to genes associated with hypoxia, oxidative stress, and inflammation, broilers fed VE supplementation in the grower diet had an 1.41 times of expression of transient receptor potential cation channel subfamily A member 1 (*TRPA1*) compared to the broilers fed supplemental n-3 fatty acids during the grower phase ($P = 0.05$). Expression of selectin E (*SELE*) was increased when broilers were fed dietary VE in the grower diet compared to the control diet ($P = 0.02$), dietary VE in the starter diet, or dietary n-3 fatty acids in the grower diet ($P = 0.01$). Extracellular matrix membrane associated gene, syndecan-4 (*SDC4*), had a 1.34 times increase in expression in p. major muscle of broilers supplemented with VE and n-3 fatty acids in the grower diet compared to the control group ($P = 0.03$). Expression of lactate dehydrogenase A (*LDHA*), an enzyme involved in glucose metabolism, expression was increased 1.17 times in the VE group supplemented during the grower phase compared to the n-3 fatty acids group supplemented during the starter phase ($P = 0.05$).

3.4 Discussion

Chapter 2 identified the effect of VE and n-3 fatty acids on phenotypic WB severity, and found that supplementation of dietary VE during the starter phase or grower phase reduced the incidence of WB myopathy. However, some birds that were phenotypically identified as normal by palpation upon microscopic evaluation of the morphological structure of the p. major muscle had damage consistent with WB (Velleman and Clark,

2015). Therefore, the present study examined the effects of dietary supplemented VE and n-3 fatty acids independently or in combination during the starter phase (0 to 10 day) or the grower phase (11 to 24 day) on p. major muscle structure and expression of genes likely associated with WB in a meat-type commercial broiler line. Specifically, the p. major muscle morphological structure and expression of genes related to muscle formation and growth, growth factors, hypoxia, oxidative stress and inflammation, extracellular matrix, cell structure and migration, calcium regulation, and glucose metabolism were examined.

More than two times more giant myofibers were identified in the broilers supplemented with VE and n-3 fatty acids during the starter phase compared to VE supplementation during the grower phase. The giant myofibers are closely related with the process of muscle growth and development. By the time of hatch, postnatal myofiber growth is dependent on a myogenic stem cell population called satellite cells as myoblasts have withdrawn from the cell cycle and muscle fiber formation is complete at hatch (Smith, 1963). Satellite cells fuse with multinucleated myofibers, donate their nuclei, and increase protein synthesis capabilities (Moss and Leblond, 1971). This results in muscle growth through hypertrophy or the enlargement of existing muscle fibers. Selection for increased breast muscle mass frequently results in large and giant myofibers, which restrict available space for the circulatory system and are thus more prone to oxidative stress associated with the WB myopathy (Dransfield and Sosnicki, 1999; Velleman et al., 2003a). This is consistent with the WB score showing that broilers supplemented with VE and n-3 fatty

acids in the starter diet had a higher severity of WB than the broilers fed VE in the grower diet.

Broilers supplemented with VE in the grower diet had the most improved muscle morphological structure. The same results were found in histological WB severity showing that VE supplementation during the grower phase had increased number of p. major muscles with no WB. This is consistent with Chapter 2 suggesting that VE supplementation reduced the incidence and severity of phenotypic WB myopathy. The improved morphology indicates that the samples had more distinct myofibers and lower level of myofiber degeneration. The degeneration process starts with disruption of sarcolemma (Straub et al., 1998). Necrosis is thereby initiated as calcium influx from the sarcoplasmic reticulum which results in activation of a calcium dependent protease called calpain (Dargelos et al., 2008). Immune cells such as heterophils and macrophages are recruited to help clear the damaged myofibers (Brigitte et al., 2010; Chazaud, 2016). This reactivates satellite cell mediated regeneration process by reentering the cell cycle, undergoing proliferation, differentiation and fusion with existing muscle fibers (Snow, 1977, 1978; Straub et al., 1998). When the satellite cells are not able to repair the damaged myofibers to its original structure, fibrosis with connective tissue replacing the myofibers is amplified and the severity of WB myopathy can be enhanced (Sihvo et al., 2014). Velleman et al. (2018) have found smaller fibers present in WB affected muscle. This is also consistent with the current study that more small myofibers were found in the n-3 fatty acid

supplementation group during the grower phase along with a higher severity of WB compared to the VE supplementation group during the starter phase.

Genes associated with muscle formation and growth factors were differentially expressed in the dietary treatments. When broilers were fed dietary VE in the grower diet, expression of *MYOD1* was increased compared to the group supplemented with VE and n-3 fatty acids in the starter diet. This indicates increased proliferation and regeneration levels in the broilers, as regeneration has been shown to result in increased expression of *MYOD1* (Füchtbauer and Westphal, 1992). Higher regeneration levels are also indicated by decreased expression of *TGFB1*, which is a strong inhibitor of myogenic proliferation and differentiation (Massague et al., 1986; Rizzino, 1988). With higher proliferation potential, satellite cell-mediated repair is likely increased leading to a reduced severity of WB. This is consistent with Velleman and Clark (2015) who reported that expression of *TGFB1* was decreased when broilers were not affected with WB myopathy compared to affected birds.

With regard to hypoxia, and oxidative stress and inflammation, expression of *TRPA1* and *SELE* were increased in the VE supplementation group during the grower phase. The *TRPA1* is a subfamily of transient receptor potential cation channel regulators of calcium level (Zurborg et al., 2007) and mediate inflammation (Bautista et al., 2006; Moilanen et al., 2012). It is activated by G protein-coupled receptors leading to increased intracellular calcium (Hardie et al., 2001; Bandell et al., 2004). Dysregulated calcium homeostasis results in degeneration (Petracci et al., 2015). The *SELE* is an endothelial leukocyte adhesion molecule (Fries et al., 1993; de Luca et al., 1994) and is involved in

chronic and acute inflammation processes in muscle (Lundberg, 2000; Ley, 2003). It is stimulated by cytokines and then recruits leukocytes to inflammatory sites (Ley et al., 1998; Patel et al., 2002). Adhesions between endothelial cells and extracellular matrix help them interact with each other and exchange information thereby improving migration and proliferation (Ruoslahti and Pierschbacher, 1987). Increased migration and proliferation are suggestive of a greater degree of myofiber regeneration. This may also explain why broilers fed additional VE in the grower diet had a reduced severity of WB as *SELE* was increased compared to the control group suggesting the presence of myofiber regeneration. Changes in expression level of genes related with myofiber regeneration and muscle ion homeostasis have been identified in Zambonelli et al. (2017). They found that the differentially expressed genes in WB affected breasts were involved in muscle development and calcium signaling pathway. Altered calcium levels have also been found in p. major muscle of 2-week-old broilers before WB phenotype has been detected at market age (Lake et al., 2019).

Expression of *SDC4* was increased in the broilers fed VE and n-3 fatty acids in the grower diet compared to the control group. Syndecan 4 is a transmembrane heparan sulfate proteoglycan and is involved in focal adhesion formation and cell migration (Longley et al., 1999; Couchman, 2003). It regulates focal adhesion by activating protein kinase C alpha through the *SDC4* cytoplasmic domain (Woods and Couchman, 1992; Lee et al., 1998; Lim et al., 2003; Shin et al., 2013). Downstream signaling is then initiated and activates Ras homolog family member A to modulate focal adhesion, cell migration, and

proliferation (Woods et al., 2000; Dovas et al., 2006). Increased expression of *SDC4* in the group supplemented with VE and n-3 fatty acids in the grower diet is suggestive of cell migration which is necessary for the repair and regeneration process.

Another differential expressed gene is *LDHA* which was increased in the broilers supplemented with VE in the grower diet compared to n-3 fatty acids supplementation in the starter diet. The *LDHA* is an enzyme regulating conversion between lactate and pyruvate (Cahn et al., 1962). Lactic acid is produced by glycolytic metabolism as p. major muscle is composed of type 2B fibers, which is an anaerobic muscle fiber. Higher muscle mass as a result of selection restricts available space for circulatory system (Dransfield and Sosnicki, 1999). With insufficient circulatory system, lactic acid cannot be removed sufficiently and will be retained in the muscle decreasing pH and result in muscle damage (Velleman et al., 2003a). Abasht et al. (2019) has identified dysregulation of lipid and glycolytic metabolism in the breast muscle of the broilers with high feed efficiency, which are more prone to have WB. Higher levels of *LDHA* has a greater potential of lactate and pyruvate conversion and thus reducing the severity of WB. This has also been identified in Zhao et al. (2020) that *LDHA* expression level was decreased in WB affected tissues.

In conclusion, VE supplementation during the grower phase (11 to 24 day) showed a beneficial effect on improving muscle morphology and reducing the severity of WB myopathy. Genes related with muscle development and growth factors, response to inflammation, extracellular matrix, and glucose metabolism were differentially expressed due to early posthatch dietary treatments. Overall, muscle morphological results are

consistent with changes in gene expression indicating a positive effect of VE supplementation during the grower phase on reducing the incidence and severity of WB myopathy. The current research represents an initial study evaluating the effect of VE and n-3 fatty acids on morphological structure of the p. major muscle and expression of genes likely associated with WB. Future research needs to be focused on determining the most beneficial supplementation concentration and administration period of VE on reducing the severity of the WB myopathy.

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Table 3.1 List of genes analyzed by Nanostring nCounter gene expression analysis

Accession number	Symbol	Gene name
Muscle formation and growth		
NM_204214.2	*MYOD1*	Myogenic differentiation 1
NM_204184.1	*MYOG*	Myogenin
NM_205065.1	*PAX7*	Paired box 7
Growth factors		
NM_001001461.1	*MSTN*	Myostatin
NM_001318456.1	*TGFB1*	Transforming growth factor beta 1
NM_205433.1	*FGF2*	Fibroblast growth factor 2
Hypoxia, oxidative stress and inflammation		
NM_204297.1	*HIF1A*	Hypoxia inducible factor 1 subunit alpha
NM_001318460.1	*TRPA1*	Transient receptor potential cation channel subfamily A member 1
NM_001163245.1	*GPX7*	Glutathione peroxidase 7
NM_204524.1	*IL1B*	Interleukin 1, beta
XM_025153162	*SELE*	Selectin E
Extracellular matrix		
XM_025144131.1	*COL1A1*	Collagen type 1 alpha 1 chain
NM_205380.2	*COL3A1*	Collagen type 3 alpha 1 chain
NM_001162399.3	*COL4A1*	Collagen type 4 alpha 1 chain
NM_001030747.2	*DCN*	Decorin
NM_001007869.1	*SDC4*	Syndecan-4
NM_001305060.2	*GPC1*	Glypican-1
Cell structure and migration		
NM_001039254.2	*ITGB1*	Integrin subunit beta 1
NM_204127.1	*ACTN1*	Actin, alpha 1
Calcium regulation		
XM_015272496.1	*RYR1*	Ryanodine receptor 1
Glucose metabolism		
NM_205284.1	*LDHA*	Lactate dehydrogenase A
Housekeeping genes		
NM_204902.2	*HMGB1*	High mobility group box 1
NM_204861.1	*ANPEP*	Alanyl aminopeptidase, membrane
NM_001007479.1	*RPL4*	Ribosomal protein L4
XM_424881.6	*FNTA*	Farnesyltransferase, CAAX box, alpha

Table 3.2 Effect of vitamin E and omega-3 fatty acids on fiber width of pectoralis major muscle of broilers

Item	Treatments[1]							SEM[2]	*P*-value
	Control	Starter VE	Starter n-3	Starter VE and n-3	Grower VE	Grower n-3	Grower VE and n-3		
Average fiber width, μm	45.87	47.54	46.55	47.90	45.36	46.75	46.75	0.42	0.71
Fiber width < 20 μm, %	6.25	4.47	6.17	5.64	5.97	6.33	6.75	0.33	0.68
20 μm ≤ Fiber width < 40 μm, %	30.41	28.41	29.62	29.34	32.04	31.74	29.43	0.70	0.83
40 μm ≤ Fiber width < 70 μm, %	55.38 [ab]	58.85 [a]	55.10 [ab]	52.46 [ab]	56.02 [ab]	51.74 [b]	54.70 [ab]	0.79	0.38
Fiber width ≥ 70 μm, %	7.96 [ab]	8.27 [ab]	9.11 [ab]	12.56 [a]	5.97 [b]	10.19 [ab]	9.12 [ab]	0.66	0.34

[a-b]Values within a row without a common letter are significantly different ($P \leq 0.05$).
[1]Broilers in the control group were fed diets with standard level of vitamin E (VE; 10 IU/kg) and omega-3 (n-3) fatty acids (n-6/n-3 ratio of 30.2:1) during the entire study (0 to 58 day). Supplementation of additional VE (200 IU/kg), n-3 fatty acids (n-6/n-3 ratio of 3.2:1), or combination of both was performed during the starter phase (0 to 10 day) or grower phase (11 to 24 day).
[2]SEM=Standard error of means.

Table 3.3 Effect of vitamin E and omega-3 fatty acids on perimysial and endomysial connective tissue space, and morphology score of pectoralis major muscle of broilers

Item	Treatments[1]							SEM[2]	*P*-value
	Control	Starter VE	Starter n-3	Starter VE and n-3	Grower VE	Grower n-3	Grower VE and n-3		
Perimysium, μm	21.97	23.37	21.46	21.09	22.34	20.33	19.9	0.77	0.93
Endomysium, μm	7.16	7.08	7.05	7.35	7.05	6.83	7.26	0.11	0.97
Morphology score[3]	2.70 [ab]	2.57 [ab]	2.68 [ab]	2.65 [ab]	2.85 [a]	2.46 [b]	2.57 [ab]	0.05	0.48

[a-b]Values within a row without a common letter are significantly different ($P \leq 0.05$).
[1]Broilers in the control group were fed diets with standard level of vitamin E (VE; 10 IU/kg) and omega-3 (n-3) fatty acids (n-6/n-3 ratio of 30.2:1) during the entire study (0 to 58 day). Supplementation of additional VE (200 IU/kg), n-3 fatty acids (n-6/n-3 ratio of 3.2:1), or combination of both was performed during the starter phase (0 to 10 day) or grower phase (11 to 24 day).
[2]SEM=Standard error of means.
[3]Scoring scale of one to five was used for pectoralis major muscle morphology evaluation. Samples with limited or no perimysial or endomysial connective tissue space, and excessive myofiber degradation were given a score of one. Samples with morphology score of five have ample perimysial and endomysial connective tissue spacing, and well-structured muscle fibers. Score of two to four are intermediate.

Table 3.4 Effect of vitamin E and omega-3 fatty acids on Wooden Breast score of pectoralis major muscle of broilers

Item	Treatments[1]							SEM[2]
	Control	Starter VE	Starter n-3	Starter VE and n-3	Grower VE	Grower n-3	Grower VE and n-3	
Wooden Breast score[3]								
Score 0, %	70.85^{b}	65.82^{b}	70.16^{b}	70.37^{b}	82.32^{a}	58.88^{b}	68.71^{b}	0.05
Score 1, %	16.75^{abcde}	26.67^{ab}	14.68^{cde}	20.38^{abcd}	7.00^{e}	27.78^{a}	21.42^{abc}	0.04
Score 2, %	8.26^{b}	4.01^{b}	14.16^{a}	4.81^{b}	3.67^{b}	8.52^{ab}	7.60^{b}	0.02
Score 3, %	4.14^{ab}	3.50^{ab}	1.00^{b}	4.44^{ab}	7.01^{a}	4.82^{ab}	2.27^{ab}	0.02

[a-e] Values within a row without a common letter are significantly different ($P \leq 0.05$).
[1] Broilers in the control group were fed diets with standard level of vitamin E (VE; 10 IU/kg) and omega-3 (n-3) fatty acids (n-6/n-3 ratio of 30.2:1) during the entire study (0 to 58 day). Supplementation of additional VE (200 IU/kg), n-3 fatty acids (n-6/n-3 ratio of 3.2:1), or combination of both was performed during the starter phase (0 to 10 day) or grower phase (11 to 24 day).
[2] SEM=Standard error of means.
[3] Wooden Breast (WB) scores were based on the degree of fibrosis, necrosis, and immune cell infiltration. Score zero = none WB, score one = mild WB, score two = moderate WB, score three = severe WB.

Table 3.5 Effect of vitamin E and omega-3 fatty acids on relative expression (fold change[1]) of genes in pectoralis major muscle of broilers

Item		Treatments[2]					
		Starter VE	Starter n-3	Starter VE and n-3	Grower VE	Grower n-3	Grower VE and n-3
Muscle formation and growth							
MYOD1	Myogenic differentiation 1	1.03	-1.01	-1.09	1.05	-1.06	1.01
MYOG	Myogenin	-1.00	1.13	1.20	1.05	1.08	1.09
PAX7	Paired box 7	1.02	1.01	1.01	-1.06	-1.07	1.05
Growth factors							
MSTN	Myostatin	1.02	-1.01	-1.02	-1.04	-1.16	-1.06
TGFB1	Transforming growth factor beta 1	-1.21	1.03	1.10	-1.27	-1.13	1.13
FGF2	Fibroblast growth factor 2	-1.02	-1.06	-1.09	-1.07	1.11	-1.05
Hypoxia, oxidative stress and inflammation							
HIF1A	Hypoxia inducible factor 1 subunit alpha	-1.01	-1.07	1.05	-1.01	-1.02	1.11
TRPA1	Transient receptor potential cation channel subfamily A member 1	-1.08	1.10	-1.12	1.13	-1.25	-1.22
GPX7	Glutathione peroxidase 7	-1.08	1.02	-1.05	-1.01	1.03	-1.07
IL1B	Interleukin 1, beta	1.05	1.01	1.17	1.09	-1.14	1.31
SELE	Selectin E	-1.07	1.06	1.03	1.29	-1.09	-1.19

Continued

Table 3.5 Continued

Item		Treatments[2]					
		Starter VE	Starter n-3	Starter VE and n-3	Grower VE	Grower n-3	Grower VE and n-3
Extracellular matrix							
COL1A1	Collagen type 1 alpha 1 chain	-1.11	-1.02	-1.11	-1.08	1.13	1.02
COL3A1	Collagen type 3 alpha 1 chain	-1.13	1.00	-1.03	-1.05	1.05	-1.02
COL4A1	Collagen type 4 alpha 1 chain	1.05	1.00	1.02	1.07	1.09	1.04
DCN	Decorin	-1.1	-1.01	-1.03	-1.12	1.02	-1.03
SDC4	Syndecan-4	-1.00	1.02	1.11	1.03	-1.09	1.34
GPC1	Glypican-1	-1.09	-1.04	1.00	-1.04	-1.03	1.01
Cell structure and migration							
ITGB1	Integrin subunit beta 1	-1.06	-1.04	-1.00	1.01	1.01	1.01
ACTN1	Actin, alpha 1	-1.21	-1.08	-1.05	-1.14	-1.14	1.13
Calcium regulation							
RYR1	Ryanodine receptor 1	1.05	1.03	-1.05	1.09	-1.03	-1.04
Glucose metabolism							
LDHA	Lactate dehydrogenase A	-1.02	-1.08	-1.12	1.08	-1.07	-1.18

[1]The fold change for each gene was calculated as the ratio between treatments and control group. If ratio is higher than 1, fold change is equal to the ratio. If ratio is lower than 1, fold change is the negative inverse of the ratio.

[2] Broilers in the control group were fed diets with standard level of vitamin E (VE; 10 IU/kg) and omega-3 (n-3) fatty acids (n-6/n-3 ratio of 30.2:1) during the entire study (0 to 58 day). Supplementation of additional VE (200 IU/kg), n-3 fatty acids (n-6/n-3 ratio of 3.2:1), or combination of both was performed during the starter phase (0 to 10 day) or grower phase (11 to 24 day).

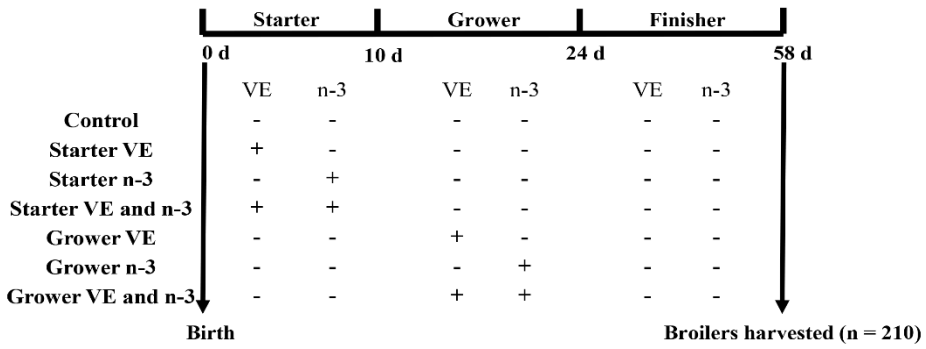

Figure 3.1 Timeline of the experimental design. Broilers in the control group were fed diets with standard level (-) of Vitamin E (VE: 10 IU/kg) and n-3 fatty acids (n-6/n-3 ratio of 30.2:1) during the entire study (0 to 58 day). Broilers in starter VE, starter n-3, and starter VE and n-3 groups were fed diets with increased level (+) of VE (200 IU/kg), n-3 fatty acids (n-6/n-3 ratio of 3.2:1), or combination of both during the starter phase (0 to 10 day). Broilers in grower VE, grower n-3, and grower VE and n-3 groups were fed diets with increased level of VE, n-3 fatty acids, or combination of both during the grower phase (11 to 24 day). All broilers were harvested at 58 days of age (n = 210).

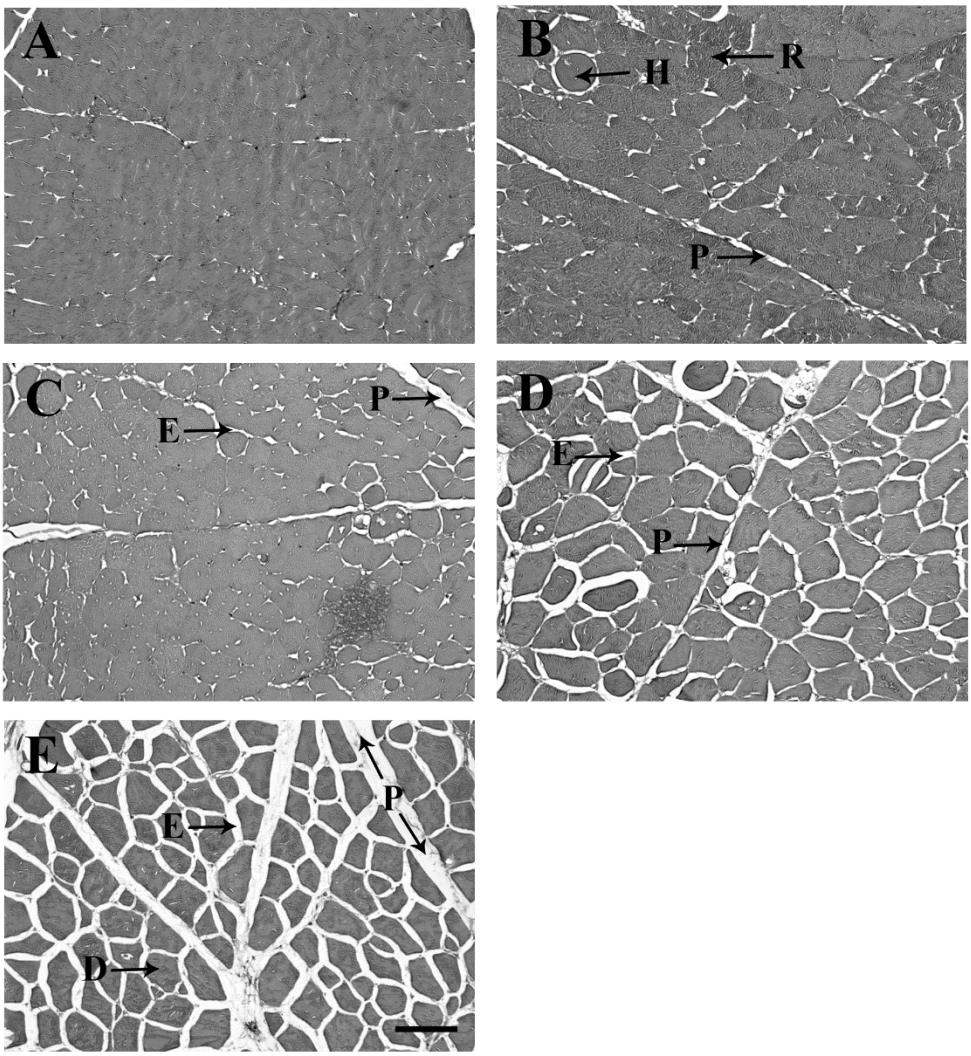

Figure 3.2 Representative photomicrographs of pectoalis major muscle with morphology score of one (A), two (B), three (C), four (D), and five (E). Samples with limited or no perimysial or endomysial connective tissue space, and excessive myofiber degradation were given a score of one. Samples with morphology score of five have ample perimysial and endomysial connective tissue spacing, and well-structured muscle fibers. Score of two to three are intermediate. H: Hypertrohic myofiber; R: Regenerating myofiber; P = Perimysial connective tissue; E = Endomysial connective tissue; D = Distinct myofiber. Scale bar = 100 μm.

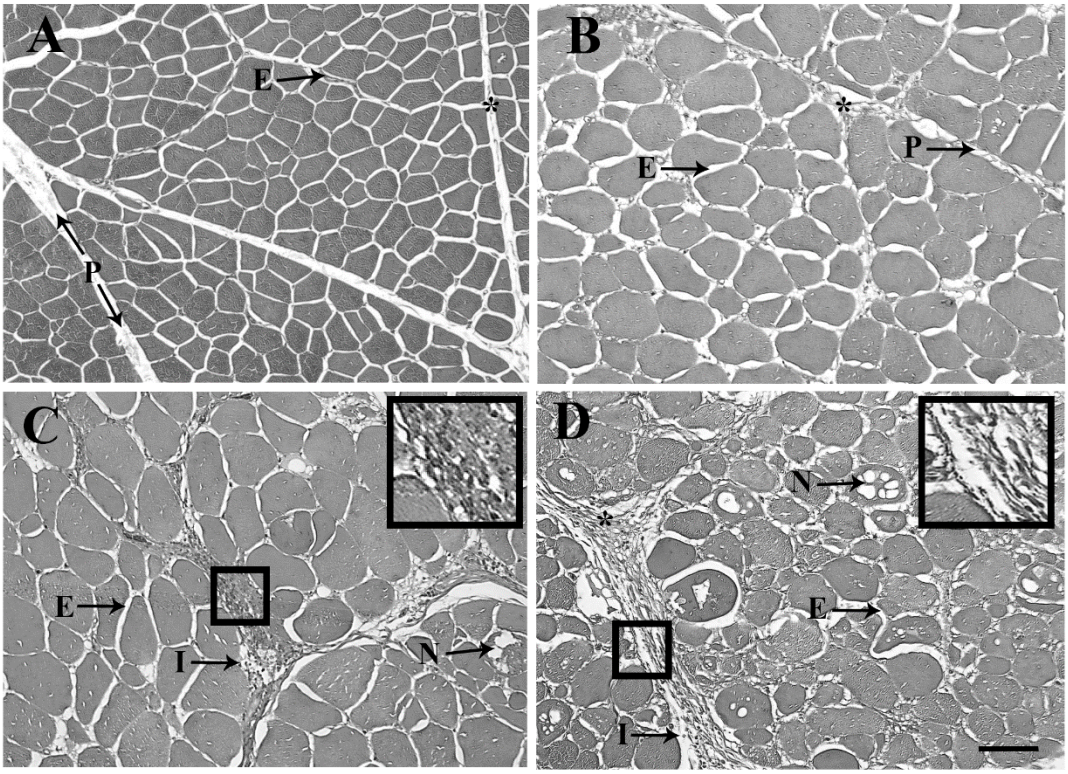

Figure 3.3 Representative photomicrographs of pectoralis major muscle samples with Wooden Breast (WB) score of zero (A), one (B), two (C), and three (D). The WB myopathy score were evaluated based on the degree of fibrosis, necrosis, and immune cell infiltration, with a score of zero representing no necrosis, fibrosis, or immune cell infiltration, a score of one representing minimal, a score of two being intermediate, and a score of three representing severe necrosis, fibrosis, and immune cell infiltration. * = Collagen; P = Perimysial connective tissue; E = Endomysial connective tissue; I = Immune cell infiltration; N = Necrotic myofibers. The boxes contain enlargements of the collagen. Scale bar = 100 μm.

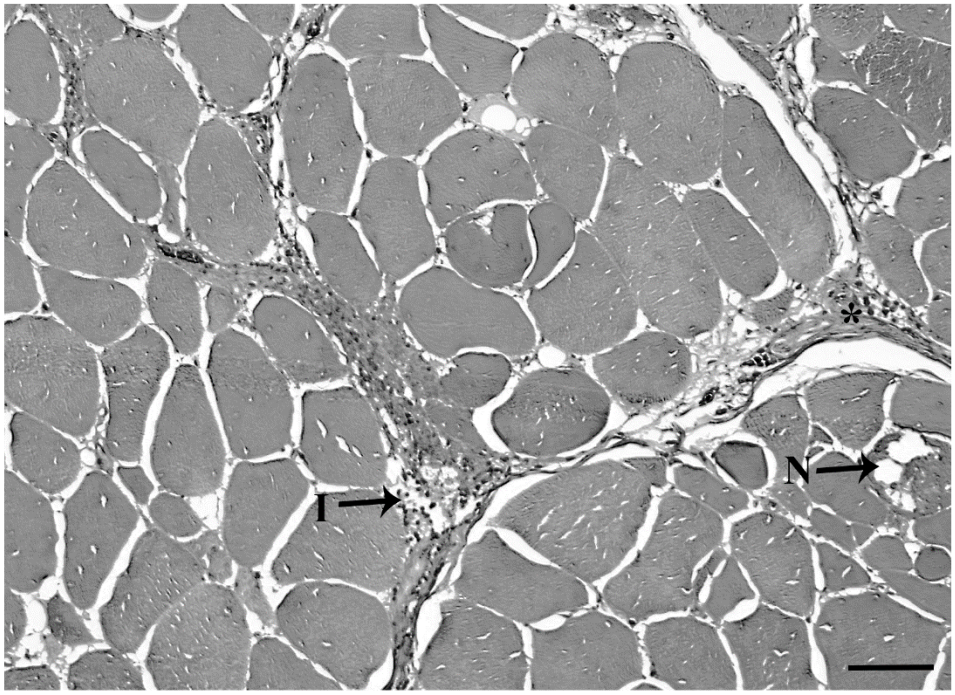

Figure 3.4 Immune cell infiltration associated with Wooden Breast myopathy. * = Collagen; I = Immune cell infiltration; N = Necrotic myofibers. Scale bar = 100 μm.

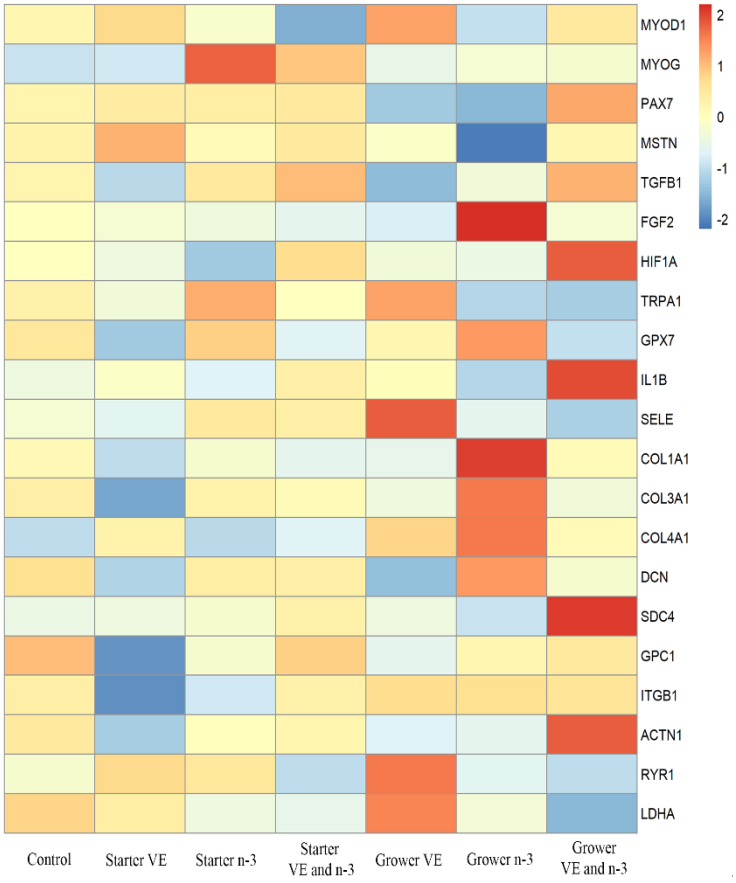

Figure 3.5 Heatmap for pectoralis major muscle of broilers with different early posthatch dietary treatments. The heatmap was generated by RStudio with the R pheatmap package (RStudio INC., Boston, MA) using the expression of each gene (in rows) and treatments (in columns). The normalized expression values are color-coded according to the legend. Broilers in the control group were fed diets with standard level of VE (10 IU/kg) and n-3 fatty acids (n-6/n-3 ratio of 30.2:1) during the entire study (0 to 58 day). Broilers in starter VE, starter n-3, and starter VE and n-3 groups were fed diets with increased level of VE (200 IU/kg), n-3 fatty acids (n-6/n-3 ratio of 3.2:1), or combination of both during the starter phase (0 to 10 day). Broilers in grower VE, grower n-3, and grower VE and n-3 groups were fed diets with increased level of VE, n-3 fatty acids, or combination of both during the grower phase (11 to 24 day). All broilers were harvested at 58 days of age (n = 210).

Chapter 4: Supplementation of Vitamin E and Omega-3 Fatty Acids During the Early Posthatch Period on Intestinal Morphology and Gene Expression in Broilers

Abstract

Early posthatch nutrition is important for gut health. Vitamin E (VE) and omega-3 (n-3) fatty acids could improve gut health through anti-oxidative and anti-inflammatory effects. The objectives of this study was to identify the effects of VE, n-3 fatty acids, and combination of both during the starter phase (0 to 10 day) or grower phase (11 to 24 day) on intestinal morphology and expression of genes associated with gut health. A total of 210 Ross 708 broilers were randomly assigned into seven treatments with 10 replicates of three birds each. The control group was fed corn-soybean meal basal diet during the entire study (0 to 58 day). Supplementation of VE (200 IU/kg), n-3 fatty acids (n-6/n-3 ratio of 3.2:1), and combination of both were fed during the starter phase (0 to 10 day) or grower phase (11 to 24 day). All of the broilers were harvested at 58 days of age. Villus height, crypt depth, villus width, distance between villi, and number of intraepithelial lymphocytes were obtained. Expression of 21 genes was measured using Nanostring analysis. Expression of solute carrier family 15 member 1 (P = 0.01) associated with peptide transport and mucin 2 (P = 0.03) related with intestinal mucus barrier was increased in the broilers supplemented with n-3 fatty acids in the grower diet compared to the control. Expression of solute carrier family 7 member 1 associated with amino acid transport was decreased in the group supplemented with n-3 fatty acids during the starter phase compared to the group

supplemented with n-3 fatty acids (P = 0.01) or VE and n-3 fatty acids during the grower phase (P = 0.03). These suggest that VE and n-3 fatty acids supplemented during the grower phase had positive effect on improving nutrient transport with n-3 fatty acids supplementation in the grower diet showing the most beneficial effect, which is critical for management intervention development to improve growth performance and meat quality in poultry industry.

4.1 Introduction

A healthy gastrointestinal system is essential for poultry growth performance and meat production (Noy and Sklan, 1998; Rinttilä and Apajalahti, 2013; Sugiharto, 2016). Various studies have suggested that early posthatch nutrition plays a key role in intestinal development (Noy et al., 2001; Batal and Parsons, 2002) and gut health (Ao et al., 2012; Jha et al., 2019). Yamauchi et al. (2016) showed that the reduced villus height and enterocytes number can be produced due to delayed feeding. In contrast, enhanced intestinal development with improved intestinal morphology has been observed with sufficient early nutrient supply (Uni and Ferket, 2003). Since the early posthatch period is important for gut health (Ao et al., 2012; Jha et al., 2019) and broiler chicks are sensitive to nutritional changes during the early posthatch period (Noy et al., 2001; Batal and Parsons, 2002), early posthatch nutritional strategies can be used to enhance gut health and nutrient absorption. With good nutrient absorption and transportation, nutrients can be utilized better in muscle growth (Hocquette et al., 1998), contributing to improved meat

production and meat quality as well as reduced development of the myopathies like Wooden Breast (WB).

Nutritional interventions including vitamin E (VE; Tappel, 1962; Burton and Traber, 1990) and omega-3 (n-3) fatty acids (Korver and Klasing, 1997; Calder, 2006; Yu et al., 2018) have the potential to lessen oxidative stress and inflammation improving gut health. Omega-3 fatty acids have been found to reduce intestinal inflammation in human (Calder, 2006; Yu et al., 2018), decrease inflammatory responses and improve immune system in broilers (Korver and Klasing, 1997; Wang et al., 2000; Saleh et al., 2009; Al-Khalifa et al., 2012; El-Katcha et al., 2014). The n-3 fatty acids, including α-linolenic acid (18:3n-3; ALA), eicosapentaenoic acid (20:5n-3; EPA), and docosahexaenoic acid (22:6n-3; DHA), are polyunsaturated fatty acids (PUFA) (Reiser, 1949). The principle link between long-chain PUFA and immune function is primarily mediated by the synthesis of oxylipins from PUFA called eicosanoids (C20-derived) or docosanoids (C22-derived) (Calder, 2003). Eicosanoids are usually synthesized by arachidonic acids (C20:4n-6) and are involved in a variety of inflammatory responses (Brock and Peters-Golden, 2007). Supplementation of n-3 fatty acids could decrease the amount of arachidonic acids and therefore decrease the amount of n-6 derived eicosanoids that are produced from arachidonic acids (Calder, 2006). Alterations in dietary n-6 and n-3 PUFA can alter eicosanoid profiles, and increased dietary n-3 PUFA can modulate production of n-6 eicosanoids by increasing n-3 derived oxylipins because the precursor PUFA (n-6 or n-3) utilize the same enzymes for synthesis. The n-3 derived eicosanoids are less biologically

potent than eicosanoids synthesized from arachidonic acids and thus present anti-inflammatory effects (Calder, 2012). In this way, n-3 fatty acids could improve gut health through reducing inflammation in the gastrointestinal tract.

Vitamin E is a widely known powerful antioxidant (Tappel, 1962; Burton and Traber, 1990). Vitamin E can protect the cell membrane from oxidation as the lipid peroxidation process is terminated (Niki, 1993). DL-α-tocopherol acetate is one of the eight forms of VE commonly used in the animal industries (Hosomi et al., 1997; Panda and Cherian, 2014). It has high biological efficiency removing free radicals in lipid peroxidation (Hosomi et al., 1997; Panda and Cherian, 2014). Dietary VE has been shown to enhance intestinal antioxidant capacity (Cheng et al., 2017) and decrease intestinal inflammatory responses (Pitargue et al., 2019) in broilers. In addition, VE and n-3 fatty acids work synergistically on reducing oxidative stress and improving immune function (Taulescu et al., 2011). Thus, VE and n-3 fatty acids are highly prone to improve gut health through anti-inflammatory and anti-oxidative effects.

Although VE (Bartov and Frigg, 1992; Rebolé et al., 2006; Lu et al., 2014) and n-3 fatty acids (Schreiner et al., 2005; Haug et al., 2007) have been evaluated in a number of studies to improve growth performance and meat quality, effects of VE and n-3 fatty acids on gut health has been barely evaluated in broilers. Therefore, the objective of the present study was to identify the effects of VE, n-3 fatty acids, and combination of both during the starter phase (0 to 10 day) or the grower phase (11 to 24 day) on intestinal morphology and

expression of genes associated with nutrient transport, hypoxia, oxidative stress, inflammation, extracellular matrix in broilers.

4.2 Materials and Methods

4.2.1 Birds and Experimental Diets

All bird protocols were approved by the Institutional Animal Care and Use Committee of The Ohio State University. A total of 210 commercial Ross 708 broiler chicks were individually wing banded and placed into pens immediately after hatch. Broilers had ad libitum access to feed and water. Birds were assigned to seven experimental groups in a completely randomized design. There were 10 pens per treatment, and each pen included three birds. The control group was fed a corn-soybean meal basal diet with VE (DL-α-tocopherol acetate, 10 IU /kg) and n-3 fatty acids (n-6/n-3 ratio of 30.2:1) at a standard level during the starter (0 to 10 day), grower (11 to 24 day), and finisher phases (25 to 58 day). Additional supplemental VE or n-3 fatty acids were fed during the starter or grower phases. For the starter dietary supplementation, starter VE, starter n-3, and starter VE and n-3 groups were fed the basal starter diet supplemented at concentrations of 200 IU/kg diet of VE, n-3 fatty acids with a n-6/n-3 ratio of 3.2:1, or a combination of both. The grower and finisher diets were the same as the control group. For the grower dietary supplementation, grower VE, grower n-3, and grower VE and n-3 groups were fed the basal grower diets supplemented at concentrations of 200 IU/kg diet of VE, n-3 fatty acids with a n-6/n-3 ratio of 3.2:1, or a combination of both. The starter and finisher diets were the

same as the control group. Diets were formulated to meet or exceed all NRC (National Research Council, 1994) nutritional requirements and recommendations in Aviagen's Ross broiler production handbook (Aviagen, 2016). Feed ingredients and nutrient composition have been previously reported in Chapter 2. At 58 days of age, all broilers were harvested in accordance with humane and commercial slaughter procedures.

4.2.2 Intestinal Morphology

To evaluate intestinal morphology, a 3 cm long section of the ileum was obtained from each broiler. Tissue samples were immediately fixed in 10% (vol/vol) buffered formalin (pH 7.0) and stored at room temperature. Histological samples were dehydrated in a graded series of alcohols, cleared in Pro Par Clearant (Anatech, Battle Creek, MI) and paraffin embedded according to the procedure of Jarrold et al. (1999). Paraffin blocks were cross sectioned at 5 µm, mounted on Starfrost Adhesive slides (Mercedes Medical, Sarasota, FL), and hematoxylin and eosin stained as described by Velleman et al. (2002). Each slide contained a minimum of four sections and imaged with a QImaging digital camera (QImaging, Burnaby, BC, Canada) attached to an Olympus IX 70 microscope (Olympus America, Mellville, NY).

Four photomicrographs from each sample were taken for measurement of villus height, crypt depth, villus width, and distance between villi. Representative images of the ileum are presented in Figure 4.1. Villus height was determined as the distance between the tip of the villi and the villus crypt junction. Crypt depth was measured as the distance of the invagination between two adjacent villi (Uni et al., 1999). Villus width was measured

at the middle part of the villi. Distance between villi was determined as the distance between the adjacent villi at the base of the villi. Measurements were taken in 10 well-structured villi and crypts from each section of each sample using Image J 1.8.0 software (National Institutes of Health, Bethesda, MD). The ratio of villus height to crypt depth was calculated as the ratio of villus height and crypt depth. Surface area of the villi was calculated as $(2\pi)\times(\text{villus width}/2)\times(\text{villus height})$ (de Los Santos et al., 2007).

Intraepithelial lymphocytes (IEL) number and epithelial cells number in villi were determined in four photomicrographs from each sample. The IEL are small round cells with nucleus centrally located and with little cytoplasm inside (Wilson et al., 1986; Figure 4.1B). Epithelial cells were counted to calculate the number of IEL per 100 epithelial cells.

4.2.3 Nanostring nCounter Gene Expression

Approximately 0.50 g of ileal mucosal scraping was isolated from the ileum and stored at -80 °C until use. Total RNA was extracted from ileal mucosal scrapings using RNAzol RT (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol. The quality and quantity of the RNA samples were checked in Molecular and Cellular Imaging Center, The Ohio State University, Wooster, OH. A total of 96 ileal samples were randomly selected and around 10 µL RNA per sample was used for gene expression analysis which was completed by Nanostring nCounter Analysis (Nanostring Technologies, Seattle, WA) following the procedure described in Geiss et al. (2008). 13 or 14 total RNA samples were randomly used for gene expression analysis from each treatment. Genes whose expression is associated with gut nutrient transport, hypoxia,

oxidative stress, inflammation, and extracellular matrix were selected as target sequences to be measured (Table 4.1). Codesets containing reporter and capture probes were designed by Nanostring Technologies. The RNA samples were hybridized to the codsets, incubated for 16 h, and digitally analyzed for quantification.

4.2.4 Statistical Analysis

Intestinal morphological attributes were analyzed as a completely randomized design using PROC MIXED procedure of SAS version 9.4 software (SAS Institute INC., Cary, NC). Individual pen was identified as the experimental unit. Dietary treatments were used as a fixed effect. Least square means were estimated with the LSMEANS procedure and separated with the PDIFF option. Significance was accepted at $P \leqslant 0.05$. Gene expression was analyzed with Nanostring nSolver version 4.0 software (Nanostring Technologies, Seattle, WA). Fold change for each gene was calculated as the ratio between each dietary treatment and the control group. If ratio was higher than one, fold change was equal to the ratio. If ratio was lower than one, fold change was the negative inverse of the ratio. Fold differences of gene expression among the dietary treatments were calculated with the fold changes. If one of the fold change was positive and another was negative, the fold difference of gene expression between the two treatments was calculated as the percentage of the multiplication of their fold changes subtract 100%. If the fold changes were both positive or negative, the fold difference of gene expression between the two treatments was calculated as the percentage of the division of their fold changes subtract 100%. A heatmap was generated with RStudio version 3.5.2 with the R pheatmap package

(RStudio INC., Boston, MA). Intestinal gene expression from Nanostring nCounter gene expression analysis were used for correlation coefficients analysis with ileal morphology. To analyze the relationship between gut health and WB, final body weight and p. major muscle weight from Chapter 2, and p. major muscle morphology and gene expression from Chapter 3 were used for correlation coefficients analysis with ileal morphology and gene expression. The final body weight, p. major muscle weight, morphology and gene expression were collected from the same broilers with the current study. Pearson correlation coefficients were determined with the CORR procedure of SAS. The $P \leqslant 0.05$ was considered as a significant difference and $P \leqslant 0.10$ was considered as a trend toward significance.

4.3 Results

4.3.1 Intestinal Morphology

Ileal morphological attributes are shown in Table 4.2. There was no significant dietary effect on villus height, crypt depth, the ratio of villus height to crypt depth, villus width, surface area of villi or IEL ($P > 0.05$). However, there was a trend that distance between villi was different among the treatments ($P = 0.08$). Broilers supplemented with n-3 fatty acids in the grower diet had a 26.0% decrease of distance between villi compared to the broilers fed dietary n-3 fatty acids in the starter diet ($P = 0.01$).

4.3.2 Nanostring nCounter Gene Expression

Heatmap analysis of gene expression from RNA extracted from ileal mucosal scrapings in different dietary treatments is shown in Figure 4.2. This data revealed differential gene expression among the treatments. Normalized gene expression abundance is color-coded according to the legend. Redness represents up-regulation of genes while blueness represents down-regulation of genes compared to the control group. Table 4.3 contains the gene fold changes. In terms of genes related with gut nutrient transport, expression of *solute carrier family 15 member 1* (*SLC15A1*; $P = 0.01$) had a 52% increase in the broilers fed dietary n-3 fatty acids in the grower diet compared to the control group. Expression of *solute carrier family 7 member 1* (*SLC7A1*) was significantly decreased in the group supplemented with n-3 fatty acids during the starter phase compared to the group of n-3 fatty acids supplementation in the grower diet ($P = 0.01$), and the group of VE and n-3 fatty acids supplementation in the grower diet ($P = 0.03$). Supplementation of VE in the grower diet had a 15.6% increase in expression of *polypeptide N-acetylgalactosaminyltransferase* 2 (*GALNT2*) compared to n-3 fatty acids supplementation in the starter diet ($P = 0.04$).

For genes associated with gut hypoxia, oxidative stress, and inflammation, expression of *mucin 2* (*MUC2*) was increased when the broilers were fed dietary n-3 fatty acids in the grower diet compared to the broilers fed with the control diet ($P = 0.03$), and n-3 fatty acids in the starter diet ($P = 0.01$). Broilers supplemented with VE during the starter phase had a 29.9% increase in expression of *interferon gamma* (*IFNG*) compared to

the broilers supplemented with n-3 fatty acids during the starter phase (P = 0.01). Vitamin E supplementation in the starter diet (P = 0.01) and grower diet (P = 0.03) decreased expression of *carbonic anhydrase 3A* (*CA3A*) in the broilers compared to VE and n-3 fatty acids supplementation during the grower phase. Supplemental VE in the grower diet had a 56.1% increase in expression of *heat shock protein family B (small) member 7* (*HSPB7*) compared to the group of n-3 fatty acids supplementation in the grower diet (P = 0.04). Expression of *collagen type 4 alpha 1 chain* (*COL4A1*), an indicator of structure of the basement membrane, had a 196% increase in the broilers fed dietary VE in the starter diet compared to the broilers supplemented with n-3 fatty acids in the grower diet (P = 0.03).

4.3.3 Correlation Coefficient of Morphology and Gene Expression

Significant correlations between ileal morphology and ileal gene expression are shown in Table 4.4. Expression of *SLC15A1* (r = 0.36, P < 0.01), *SLC5A1* (r = 0.23, P = 0.03), and *MUC2* (r = 0.30, P < 0.01) was positively correlated with villus height. Expression of *HSPB7* was positively correlated with crypt depth (r = 0.26, P = 0.01). Expression of *SLC15A1* (r = 0.31, P < 0.01), *SLC5A1* (r = 0.26, P = 0.01), *GALNT2* (r = 0.25, P = 0.01), and *MUC2* (r = 0.30, P < 0.01) was positively correlated and *HSPB7* (r = -0.35, P < 0.01) was negatively correlated with the ratio of villus height to crypt depth. There was a trend of positive correlation between surface area and expression of *SLC15A1* (r = 0.17, P = 0.09). Meanwhile, IEL was positively correlated with expression of *IFNG* (r = 0.25, P = 0.02).

Correlation coefficients between ileal morphology and gene expression, and broiler final body weight, p. major muscle weight, morphology and gene expression are shown in Table 4.5. Broiler final body weight, p. major muscle weight, and p. major muscle fiber width were positively correlated with villus height, the ratio of villus height to crypt depth, and surface area ($P \leqslant 0.05$). Broiler final body weight (r = -0.21, P = 0.04) and p. major muscle weight (r = -0.20, P = 0.05) were negatively correlated with ileal *HSPB7* expression. The p. major muscle fiber width was positively correlated with ileal *COL4A1* expression (r = 0.23, P = 0.02) and *MUC2* expression (r = 0.20, P = 0.05). Morphology score, which was obtained with higher score representing more well-structured muscle fibers, was negatively correlated with IEL (r = -0.21, P = 0.04) and positively correlated with *MUC2* expression (r = 0.21, P = 0.04). Expression of *COL4A1* in p. major muscle was negatively correlated with ileal IEL (r = -0.21, P = 0.05) and ileal *HSPB7* expression (r = -0.23, P = 0.02).

4.4 Discussion

Early posthatch period is essential for intestinal development (Noy et al., 2001; Uni et al., 2003). Broiler chicks are sensitive to nutrition during this period in which sufficient early nutrient supply improves intestinal morphology and enhances intestinal development (Noy et al., 2001; Batal and Parsons, 2002; Ao et al., 2012; Jha et al., 2019). With good intestinal development, broilers have improved nutrient absorptive functions and gut health promoting animal growth including muscle growth and potentially decrease muscle

disorders (Jha et al., 2019) such as WB. Nutritional interventions targeting reducing oxidative stress such as VE, and reducing inflammation such as n-3 fatty acids during the early posthatch period will potentially influence gut health as well as alter nutrient absorption in small intestine. Thus, in the current study, intestinal morphology and expression of genes associated with gut nutrient transport, hypoxia, oxidative stress, inflammation, and extracellular matrix in the ileal mucosa of broilers supplemented with VE and n-3 fatty acids independently or in combination during the starter phase (0 to 10 day) or grower phase (11 to 24 day) were investigated.

Intestinal morphology plays a crucial role in indicating gut health in the broilers. Villus height, crypt depth, villus width, surface area of the villi, and distance between villi can be used to evaluate the integrity and nutrient absorption of the gastrointestinal system (Wright, 1981; Xu et al., 2003). There was no significant difference in most of the morphological attributes. However, supplemental n-3 fatty acids in the grower diet has a trend to decrease distance between adjacent villi compared to the group of n-3 fatty acids supplementation during the starter phase. Decreased distance between villi represents improved intestinal morphology (Uni et al., 2001; Azevedo et al., 2020), which could be more effective for nutrient absorption due to a shorter distance of nutrients travelling and diffusion of the nutrients. Along with the intestinal morphology, expression of *SLC7A1* and *MUC2* was higher in the group supplemented with n-3 fatty acids in the grower diet than in the starter diet. The *SLC7A1*, also called cationic amino acid transporter-1, is essential in transferring cationic amino acid from enterocyte to blood for circulation (Devés

and Boyd, 1998). The mucus layer in the intestinal epithelium is mainly composed of mucin, which is synthesized by goblet cells (Deplancke and Gaskins, 2001). Mucin 2 is the main gel-forming mucin providing the protective barrier protecting the ileal epithelium against antigens (Velcich et al., 2002). It is widely recognized as a marker of gut health in poultry (Forder et al., 2012; Wei et al., 2012; Li et al., 2015). Absorption and immune function could be affected by mucin deficiency (Uni et al., 2003). Meanwhile, n-3 fatty acids supplementation during the grower phase increased expression level of *SLC15A1* and *MUC2* compared to the control group. *SLC15A1*, a peptide transporter, is a key transporter of dipeptides and tripeptides in the enterocytes (Osmanyan et al., 2018). Expression level of *SLC15A1* is closely related with protein synthesis and degradation (Gaildrat et al., 2005). Therefore, decreased distance between villi and increased gene expression levels indicate that supplementation of n-3 fatty acids during the grower phase showed a more beneficial effect on improving gut health than supplementation during the starter phase. The intestinal mucus barrier and nutrient transport would be enhanced by n-3 fatty acids supplementation in the grower diet.

In terms of differentially expressed genes in VE and n-3 fatty acids combined supplementation group, expression of *SLC7A1* was increased when broilers were fed dietary VE and n-3 fatty acids in the grower phase compared to the n-3 fatty acids supplementation group during the starter phase. Additionally, expression of *CA3A* was decreased in the VE and n-3 fatty acids group supplemented during the grower phase compared to the VE group supplemented during the starter phase or the grower phase. Most

carbonic anhydrases are efficient enzymes catalyzing the reversible hydration reaction of carbon dioxide (Breton, 2001). The CA3A has also shown an effect on fatty acids metabolism converting acetyl-CoA into malonyl-CoA (Alver et al., 2004). Lipid metabolism has been found to modulate macrophage response by triggering pro-inflammatory activities (Riera-Borrull et al., 2017). Therefore, supplementation of VE and n-3 fatty acids during the grower phase may be associated with improved nutrient absorption, amino acid transport, altered lipid synthesis, and reduced pro-inflammatory activities in small intestines. The beneficial changes in the group supplemented with VE and n-3 fatty acids during the grower phase are consistent with the WB incidence rate in Chapter 2. Supplementation of VE and n-3 fatty acids in the grower diet had a 36% reduction in moderate WB incidence compared to the control group. This suggests that improvement in gut health and nutrient absorption could be related with the reduced muscle disorders incidence.

Another differentially expressed gene is *COL4A1*, which has an increased expression in the broilers supplemented with VE in the starter diet compared to n-3 fatty acids supplementation during the grower phase. Collagen type IV is a critical component of gut basement membrane (Zhang et al., 2003). Basement membrane has sheet-like structure acting as a barrier separating extracellular matrix and epithelial cells (Glanville, 1987). Epithelial dysfunction could be produced when the basement membrane is altered (Groulx et al., 2011). Higher expression of *COL4A1* in the group supplemented with VE during the starter phase indicates improved integrity of basement membrane.

Correlation coefficient analysis comparing intestinal morphology to intestinal gene expression showed that *SLC15A1*, *SLC5A1*, *GALNT2*, and *MUC2* were positively correlated with villus height or the ratio of villus height to crypt depth. Although the correlations were not strong, these correlations could be beneficial for understanding the relationship between intestinal morphology and gene expression differentiation. The *SLC15A1* is responsible for dipeptides and tripeptides transport in the enterocytes (Osmanyan et al., 2018). The *SLC5A1* is a sodium glucose cotransporter transporting glucose from the intestine (Wright, 2013). The *GALNT2* encodes polypeptide N-acetylgalactosaminyltransferase 2, which transfers N-acetylgalactosamine to serine or threonine residue during the biosynthesis of O-linked oligosaccharide (Ten Hagen et al., 2003). The positive correlation between ileal morphology and expression of the gene involved in nutrient transport suggests that intestinal nutrient transport are closely associated with ileal structure. Meanwhile, *GALNT2* is also involved in glycosylation of mucin (Ten Hagen et al., 2003), which is consistent with current study that *GALNT2* and *MUC2* has similar correlation pattern with the ileal morphology. There was a positive correlation between expression of *IFNG* and IEL. Both *IFNG* and IEL can indicate inflammatory state in gastrointestinal system. The IFNG can be produced by T cells, macrophages, natural killer cells, and mucosal epithelial cells (Adams, 1989; Schroder et al., 2004). It plays a variety of roles in innate and adaptive immune responses (Young, 1996; Bach et al., 1997). Additionally, *HSPB7* was negatively correlated with the ratio of villus height to crypt depth. The *HSPB7* is a member of heat-shock protein family (Vos et

al., 2009). It is involved in responses to oxidative stress by activating nuclear factor erythroid 2-related factor 2 signaling pathway (Sun et al., 2019). These genes associated with oxidative stress and inflammation were correlated with ileal morphology, indicating that intestinal oxidative stress and inflammation status can affect intestinal structure and function.

Ileal morphology and gene expression were correlated with broiler final body weight, p. major muscle weight, morphology and gene expression. Ileal villus height, the ratio of villus height to crypt depth, and surface area were positively correlated with broiler final body weight, p. major muscle weight, and p. major muscle fiber width. Ileal *COL4A1* and *MUC2* were positively correlated with p. major muscle fiber width. These could be suggestive that improved intestinal structure would have positive influence on intestinal nutrient absorption, thereby have beneficial effect on growth performance and breast muscle growth. This is consistent with Sugiharto (2016) that a well-functioned gastrointestinal system is an important factor to promote broiler growth performance. On the other hand, *HSPB7*, the expression of which would be up regulated in responses to oxidative stress (Sun et al., 2019), was negatively correlated with final body weight and p. major muscle weight. This showed that intestinal oxidative stress would detrimentally affect broiler body weight and breast muscle growth. The positive correlation between ileal *MUC2* and p. major muscle morphology score indicates that intestinal structure is closely related with p. major muscle structure. The p. major muscle morphology score and expression of *COL4A1* in p. major muscle were negatively correlated with ileal IEL. The

IEL serves as a critical indicator of inflammatory state in intestinal immune system and performs a variety of immune functions (Yamamoto et al., 1998; Kakar et al., 2003). They are located in the epithelial layer of the intestine and can release cytokines to facilitate killing mechanisms once they sense the antigen in the gut (Kakar et al., 2003; Rieger et al., 2015). In addition, expression of *COL4A1* in p. major muscle was negatively correlated with ileal *HSPB7*. The p. major muscle morphology score evaluation used a one to five scoring scale with score of one representing limited or no perimysial or endomysial connective tissue space and excessive myofiber degradation, and a score of five having ample perimysial and endomysial connective tissue spacing, and well-structured muscle fibers. The negative correlations suggest that broilers with lower level of ileal oxidative stress and inflammation would have more well-structured breast muscle fibers as well as improved breast muscle basement membrane. The p. major muscle growth could be closely related with intestinal inflammation and oxidative stress levels.

In conclusion, supplementation of VE and n-3 fatty acids independently and in combination showed a more beneficial effect on improving intestinal morphology during the grower phase than the starter phase. Genes involved in gut nutrient transport, oxidative stress, and inflammation were differentially expressed in the broilers supplemented with VE, n-3 fatty acids, or combination of both during the grower phase. Intestinal morphological results were consistent with changes in gene expression indicating a positive effect of VE and n-3 fatty acids supplementation during the grower phase on improving gut health and nutrient transport, with supplementation of n-3 fatty acids during the grower

phase showing the most beneficial effects. The current study implies that supplementation of VE and n-3 fatty acids during the grower phase could enhance intestinal nutrient transport, which would be beneficial for broiler growth performance and meat quality. Future research needs to be focused on determining the most beneficial supplementation concentration and administration period to improve gut health in the broilers.

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Table 4.1 List of genes analyzed by Nanostring nCounter gene expression analysis

Accession number	Symbol	Gene full name
Gut nutrient transport		
NM_204365	*SLC15A1*	Solute carrier family 15 member 1
XM_004935370.3	*SLC3A1*	Solute carrier family 3 member 1
NM_001145490.1	*SLC7A1*	Solute carrier family 7 member 1
XM_425011.5	*SLC1A3*	Solute carrier family 1 member 3
NM_001293240.1	*SLC5A1*	Solute carrier family 5 member 1
NM_207178.1	*SLC2A2*	Solute carrier family 2 member 2
XM_025142667	*SLC2A5*	Solute carrier family 2 member 5
NM_001007923.1	*FABP2*	Fatty acid binding protein 2
XM_015284386.2	*GALNT2*	Polypeptide N-acetylgalactosaminyltransferase 2
Gut hypoxia, oxidative stress and inflammation		
NM_001318434.1	*MUC2*	Mucin 2
NM_205149.1	*IFNG*	Interferon gamma
NM_204267.1	*LITAF*	Lipopolysaccharide induced TNF factor
NM_001282432.1	*CXCR1*	C-X-C motif chemokine receptor 1
NM_001123031.1	*CRH*	Corticotrophin releasing hormone
XM_427836.6	*HSPB7*	Heat shock protein family B (small) member 7
NM_001163245.1	*GPX7*	Glutathione peroxidase 7
NM_001115017.4	*SELENOO*	Selenoprotein O
NM_001277411.1	*CA3A*	Carbonic anhydrase 3A
NM_204524.1	*IL1B*	Interleukin 1, beta
XM_025153162	*SELE*	Selectin E
Extracellular matrix		
NM_001162399.3	*COL4A1*	Collagen type 4 alpha 1 chain
Housekeeping genes		
NM_204902.2	*HMGB1*	High mobility group box 1
NM_204861.1	*ANPEP*	Alanyl aminopeptidase, membrane
NM_001007479.1	*RPL4*	Ribosomal protein L4
XM_424881.6	*FNTA*	Farnesyltransferase, CAAX box, alpha

Table 4.2 Effect of vitamin E and omega-3 fatty acids on ileal morphology of broilers

	Treatments[1]							SEM[2]	*P*-value
	Control	Starter VE	Starter n-3	Starter VE and n-3	Grower VE	Grower n-3	Grower VE and n-3		
Villus height (μm)	1241.12	1241.73	1263.05	1235.35	1239.9	1262.41	1253.86	20.86	0.99
Crypt depth (μm)	276.36	274.87	275.80	289.37	271.76	286.25	303.21	3.26	0.19
Villus/crypt[3]	4.60	4.62	4.64	4.50	4.70	4.53	4.22	0.07	0.71
Villus width (μm)	281.83	275.88	278.40	287.85	261.50	258.79	263.02	3.22	0.21
Surface area (mm^2)	1.10	1.09	1.12	1.13	1.02	1.06	1.04	0.02	0.79
Distance between villi (μm)	54.34	55.56	63.96	56.01	60.07	50.78	57.27	1.08	0.08
IEL[4]	25.85	24.69	24.36	25.09	21.65	24.61	21.29	0.61	0.24

[1]Broilers in control group were fed diets with standard level of vitamin E (VE; 10 IU/kg) and omega-3 (n-3) fatty acids (n-6/n-3 ratio of 30.2:1) during the entire study (0 to 58 day). Supplementation of dietary VE (200 IU/kg), n-3 fatty acids (n-6/n-3 ratio of 3.2:1), or combination of both were fed during the starter phase (0 to 10 day) or grower phase (11 to 24 day).
[2]SEM=Standard error of the mean.
[3]Villus/crypt=Ratio of villus height to crypt depth.
[4]IEL=Number of intraepithelial lymphocyte per 100 epithelial cells.

Table 4.3 Effect of vitamin E and omega-3 fatty acids on ileal relative gene expression[1]

Item		Treatments[2]					
		Starter VE	Starter n-3	Starter VE and n-3	Grower VE	Grower n-3	Grower VE and n-3
Gut nutrient transport							
SLC15A1	Solute carrier family 15 member 1	1.26	1.28	1.47	1.35	1.52	1.13
SLC3A1	Solute carrier family 3 member 1	1.08	-1.03	1.19	1.07	1.23	-1.04
SLC7A1	Solute carrier family 7 member 1	-1.01	-1.21	1.04	1.13	1.08	1.00
SLC1A3	Solute carrier family 1 member 3	1.24	1.04	1.13	1.37	1.07	1.32
SLC5A1	Solute carrier family 5 member 1	1.11	-1.09	1.01	1.12	1.22	1.02
SLC2A2	Solute carrier family 2 member 2	1.53	1.23	-1.41	1.1	1.24	-1.16
SLC2A5	Solute carrier family 2 member 5	1.16	1.13	1.15	1.16	1.21	1.15
FABP2	Fatty acid binding protein 2	1.14	1.18	1.12	1.01	1.2	-1.00
GALNT2	Polypeptide N-acetylgalactosaminyltransferase 2	-1.01	-1.08	1.02	1.07	1.01	-1.03
Gut hypoxia, oxidative stress and inflammation							
MUC2	Mucin 2	1.06	-1.08	1.27	1.17	1.41	-1.03

Continued

Table 4.3 Continued

Item		Treatments[2]					
		Starter VE	Starter n-3	Starter VE and n-3	Grower VE	Grower n-3	Grower VE and n-3
IFNG	Interferon gamma	1.11	-1.17	1.03	1.22	1.14	1.04
LITAF	Lipopolysaccharide induced TNF factor	1.02	-1.06	1.07	1.08	1.17	-1.03
CXCR1	C-X-C motif chemokine receptor 1	1.03	-1.07	-1.05	1.12	1.06	1.04
CRH	Corticotrophin releasing hormone	-1.01	-1.13	1.19	1.11	1.25	-1.06
HSPB7	Heat shock protein family B member 7	-1.2	-1.14	-1.38	1.02	-1.53	-1.18
GPX7	Glutathione peroxidase 7	1.06	1.01	-1.04	1.08	-1.09	-1.04
SELENOO	Selenoprotein O	-1.02	-1.01	-1.01	1	-1.01	-1.09
CA3A	Carbonic anhydrase 3A	1.29	1.01	1.09	1.27	1.06	-1.21
IL1B	Interleukin 1, beta	1.05	-1.05	-1.12	-1.01	-1.03	1.24
SELE	Selectin E	1.08	1.06	-1.09	1.13	-1.13	-1.09
Extracellular matrix							
COL4A1	Collagen type 4 alpha 1 chain	1.62	1.29	1.06	-1.68	-1.83	1.30

[1]The fold change for each gene was calculated as the ratio between treatments and the control group. If the ratio was higher than one, fold change is equal to the ratio. If the ratio was lower than one, fold change is the negative inverse of the ratio.
[2]Broilers in the control group were fed diets with standard level of vitamin E (VE; 10 IU/kg) and omega-3 (n-3) fatty acids (n-6/n-3 ratio of 30.2:1) during the entire study (0 to 58 day). Supplementation of dietary VE (200 IU/kg), n-3 fatty acids (n-6/n-3 ratio of 3.2:1), or combination of both were performed during the starter phase (0 to 10 day) or grower phase (11 to 24 day).

Table 4.4 Correlation coefficients for ileal morphology and gene expression[1]

	SLC15A1[2]	*SLC5A1*[3]	*GALNT2*[4]	*MUC2*[5]	*IFNG*[6]	*HSPB7*[7]
Villus height (μm)						
Pearson	0.36	0.23	0.17	0.30	0.16	-0.19
P-value[8]	< 0.01	0.03	0.10	< 0.01	0.14	0.07
Crypt depth (μm)						
Pearson	0.07	0.04	-0.11	0.02	0.17	0.26
P-value	0.48	0.69	0.29	0.83	0.09	0.01
Villus/crypt[9]						
Pearson	0.31	0.26	0.25	0.30	0.17	-0.35
P-value	< 0.01	0.01	0.01	< 0.01	0.10	< 0.01
Surface area (mm^2)						
Pearson	0.17	0.11	-0.04	0.11	0.04	-0.11
P-value	0.09	0.30	0.67	0.28	0.69	0.30
IEL[10]						
Pearson	0.07	0.14	0.06	0.04	0.25	0.04
P-value	0.49	0.18	0.56	0.67	0.02	0.68

[1]Perason correlation coefficient for ileal morphology (in rows) and expression level of differentially expressed genes (in columns).
[2]*SLC15A1* = Solute carrier family 15 member 1.
[3]*SLC5A1* = Solute carrier family 5 member 1.
[4]*GALNT2* = Polypeptide N-acetylgalactosaminyltransferase 2.
[5]*MUC2* = Mucin 2, oligomeric mucus/gel-forming.
[6]*IFNG* = Interferon gamma.
[7]*HSPB7* = Heat shock protein family B member 7.
[8]*P*-value for each Pearson correlation coefficient.
[9]Villus/crypt=Ratio of villus height to crypt depth.
[10]IEL=Number of intraepithelial lymphocytes per 100 epithelial cells.

Table 4.5 Correlation coefficients for ileal morphology and gene expression, and broiler final body weight, pectoralis major muscle weight, morphology and gene expression[1]

	Final body weight	P. major muscle weight	P. major muscle fiber width	Morphology score[2]	*COL4A1*[3]
Villus height					
Pearson	0.32	0.22	0.24	-0.16	-0.13
P-value[4]	< 0.01	0.03	0.02	0.12	0.21
Villus/crypt[5]					
Pearson	0.30	0.23	0.28	-0.19	-0.10
P-value	< 0.01	0.02	0.01	0.06	0.35
Surface area					
Pearson	0.28	0.22	0.27	-0.15	-0.01
P-value	0.01	0.03	0.01	0.15	0.96
IEL[6]					
Pearson	0.08	0.07	0.17	-0.21	-0.21
P-value	0.46	0.50	0.10	0.04	0.05
COL4A1					
Pearson	-0.16	-0.19	0.23	0.02	-0.14
P-value	0.12	0.07	0.02	0.84	0.17
HSPB7[7]					
Pearson	-0.21	-0.20	0.02	0.17	-0.23
P-value	0.04	0.05	0.86	0.09	0.02
MUC2[8]					
Pearson	0.04	0.02	0.20	0.21	0.10
P-value	0.72	0.86	0.05	0.04	0.34

[1]Perason correlation coefficient for ileal morphology and differentially expressed genes (in rows) and pectoralis major muscle (p. major muscle; breast muscle) weight, morphology and differentially expression genes (in columns).
[2]Scoring scale of one to five was used for pectoralis major muscle morphology evaluation. Samples with limited or no perimysial or endomysial connective tissue space, and excessive myofiber degradation were given a score of one. Samples with morphology score of five have ample perimysial and endomysial connective tissue spacing, and well-structured muscle fibers. Score of two to four are intermediate.
[3]*COL4A1* = Collagen type 4 alpha 1 chain; [4]*P*-value for each Pearson correlation coefficient; [5]Villus/crypt=Ratio of villus height to crypt depth; [6]IEL=Number of intraepithelial lymphocytes per 100 epithelial cells; [7]*HSPB7* = Heat shock protein family B member 7; [8]*MUC2* = Mucin 2, oligomeric mucus/gel-forming.

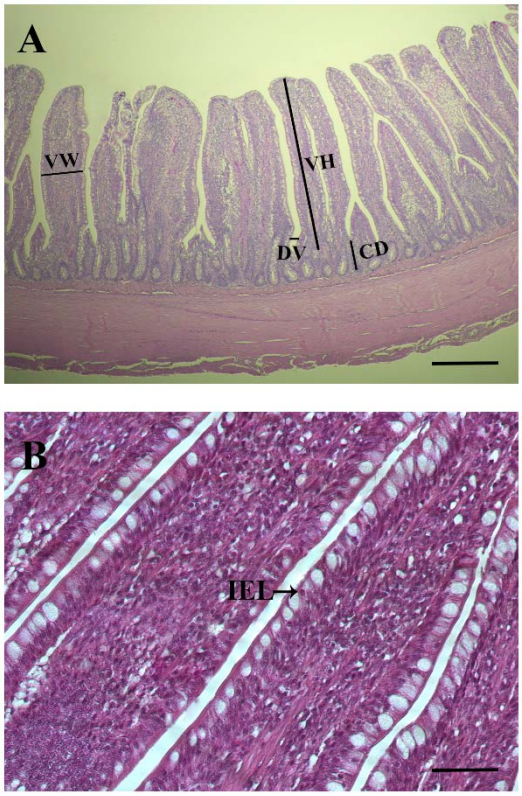

Figure 4.1 Representative photomicrographs of the ileum of the broilers. Figure A shows measurements of villus height, crypt depth, villus width, and distance between villi. Villus height was determined as the distance between the tip of the villi and the villus crypt junction. Crypt depth was measured as the distance of the invagination between two adjacent villi. Villus width was measured at the middle part of the villi. Distance between villi was determined as the distance between the adjacent villi at the base of the villi. Measurements were taken in 10 well-structured villi and crypts from each section of each sample. Scale bar is 100 μm. Figure B shows intraepithelial lymphocytes centrally located in epithelial cells. VH = Villus height; CD = Crypt depth; VW = Villus width; DV = Distance between villi; IEL = Intraepithelial lymphocyte. Scale bar is 50 μm.

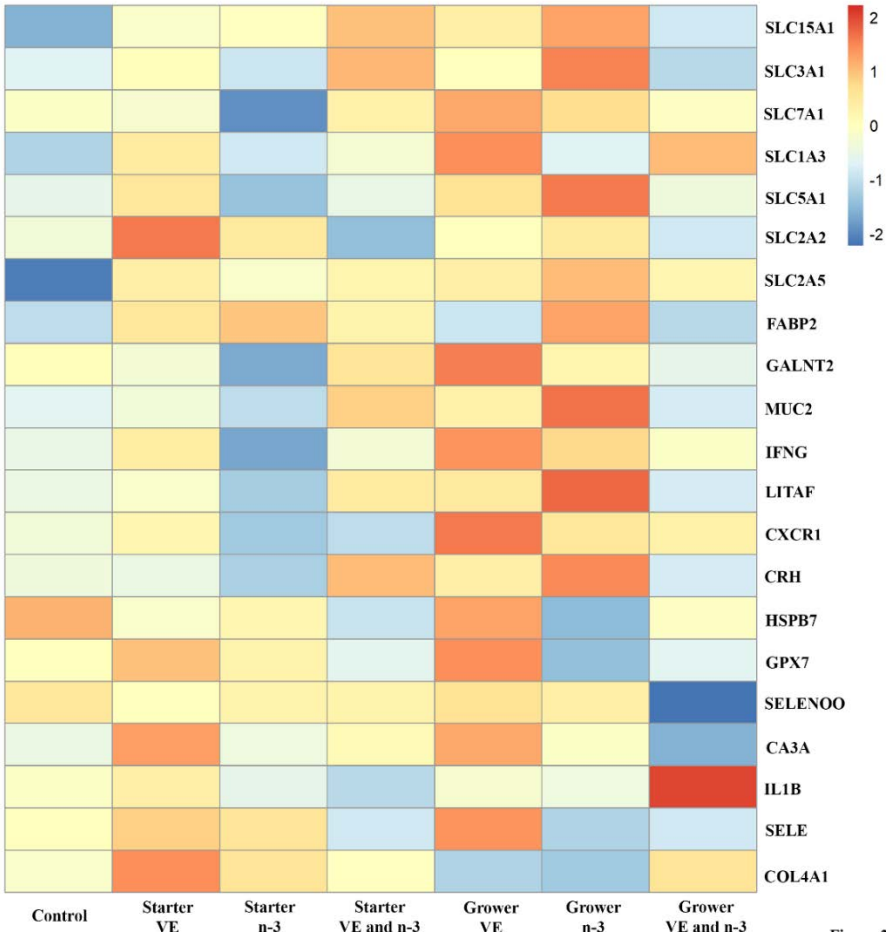

Figure 4.2 Heatmap for gene expression in ileal mucosal scrapings from broilers with different early posthatch dietary treatments. The heatmap has the expression of each gene (in rows) and treatments (in columns). The normalized expression levels are color-coded according to the legend. Redness represents up-regulation of genes while blueness represents down-regulation of genes compared to the control group. Broilers in control group were fed diets with standard level of vitamin E (VE; 10 IU/kg) and omega-3 (n-3) fatty acids (n-6/n-3 ratio of 30.2:1) during the entire study (0 to 58 day). Supplementation of dietary VE (200 IU/kg), n-3 fatty acids (n-6/n-3 ratio of 3.2:1), or combination of both were fed during the starter phase (0 to 10 day) or grower phase (11 to 24 day). Ileal mucosal scrapings were collected when the broilers were harvested at 58 days of age.

Chapter 5: Effect of vitamin E and alpha lipoic acid on the development of the Wooden Breast myopathy in broilers

Abstract

Wooden Breast (WB) is characterized by a rigid pectoralis major (p. major) muscle that is under severe oxidative stress and inflammation. The objective of the study was to identify the effects of antioxidants vitamin E (VE) and alpha lipoic acid (ALA) with anti-inflammatory effects on the developmental onset, severity, and progression of WB based on p. major muscle morphology and expression of genes associated with WB in broilers during the first 3 weeks posthatch. A total of 160 newly hatched Ross 708 broiler chicks were randomly assigned into a control group and three dietary treatments with 10 replicates of four birds each. Supplementation of VE (160 mg/kg) and ALA (500 mg/kg) independently and in combination were fed during the first 3 weeks posthatch. At 1, 2 and 3 weeks of age, one chick from each pen was harvested. Growth performance and phenotypic WB score were obtained. Microscopic assessment of p. major muscle included myofiber width, perimysial/endomysial connective tissue spacing, morphology score, and microscopic WB score. Expression of genes associated with muscle formation and growth, adipogenesis, extracellular matrix, oxidative stress and inflammation were measured by real-time quantitative PCR. There was no phenotypic detection of WB by palpation during the course of the study. In contrast, microscopic changes associated with WB was detected beginning at 1 week of age in all groups. Supplementation of VE and ALA independently

and in combination reduced microscopic WB severity at 2 and 3 weeks of age compared to the control group. Expression of *myogenic determination factor 1* and *peroxisome proliferator-activated receptor gamma* was reduced in all dietary treatments compared to the control at 3 weeks of age ($P \leqslant 0.05$), suggestive of reduced muscle degeneration and lipid deposition. These data suggest that VE and ALA supplemented independently and in combination had positive effects on mitigating WB severity as early as 2 weeks of age, which will likely decrease the severity of phenotypic WB at market age.

5.1 Introduction

Broiler breast muscle myopathies have arisen in recent years especially in broilers with higher growth rates and breast muscle yield (Brewer et al., 2012; Tijare et al., 2016). Among the myopathies, Wooden Breast (WB) is of great concern as it has been reported worldwide (Sihvo et al., 2014; Kuttappan et al., 2016; Xing et al., 2019). The WB resulted in an economic loss of over $200 million in 2016 due to a palpable hard breast muscle frequently leading to the condemnation of the breast meat (Sihvo et al., 2014; Kuttappan et al., 2016). Wooden Breast is phenotypically characterized by palpation of a rigid pectoralis major (p. major: breast muscle) muscle (Sihvo et al., 2014) and histologically characterized by myodegeneration including myofiber necrosis (Papah et al., 2017), fibrosis (Sihvo et al., 2014; Velleman and Clark, 2015), and immune cell infiltration (Sihvo et al., 2014, 2017). Recent studies have linked WB to oxidative stress (Mutryn et al., 2015; Abasht et al., 2016; Brothers et al., 2019), inflammation (Mutryn et al., 2015; Zambonelli

et al., 2017), and dysregulation of lipid metabolism (Abasht et al., 2019; Brothers et al., 2019; Lake and Abasht, 2020). Genes involved in oxidative stress and inflammation such as selectin E (*SELE*) (Chapter 3), and lipid metabolism such as peroxisome proliferator-activated receptor gamma (*PPARγ*) and CCAAT/enhancer binding protein alpha (*CEBPα*) (Brothers et al., 2019; Lake et al., 2019) have been shown to be differentially expressed in WB affected breast muscle compared to non-affected breast muscle.

Wooden Breast has been identified in broilers as early as 18 days of age and the severity of WB increases with age and growth of the birds (Sihvo et al., 2017). The development of WB is closely associated with skeletal muscle growth. During the posthatch period, muscle growth is dependent on the adult myoblast, satellite cells, by fusing with existing myofibers which leads to muscle growth through hypertrophy (Stockdale and Holtzer, 1961; Moss and Leblond, 1971). The satellite cells require the expression of myogenic transcriptional regulatory factors such as myogenic determination factor 1 (MyoD) and myogenin (MyoG) for their proliferation and differentiation (Brunetti and Goldfine, 1990; Yablonka-Reuveni et al., 1999). One of the transmembrane extracellular matrix heparan sulfate proteoglycans, syndecan-4 (SDC4), plays an essential role in muscle growth and development through regulating satellite cell proliferation, migration, and differentiation (Velleman et al., 2007; Shin et al., 2013). Satellite cell mitotic activity is highest in the first week after hatch (Halevy et al., 2000) and can be affected by nutrition (Halevy et al., 2000; Velleman et al., 2010; Powell et al., 2014). Since satellite cells have their maximal mitotic activity immediately after hatch and are sensitive

to nutrition during this time, nutritional strategies during the immediate posthatch period can likely be used to influence breast muscle development and growth and reduce the incidence of WB.

Vitamin E (VE) and alpha lipoic acid (ALA) are two nutrients that have powerful antioxidant capacities (Rymer and Givens, 2010; Cheng et al., 2016; El-Senousey et al., 2018). They can terminate lipid peroxidation process by reacting with free radicals and therefore mitigate cell membrane oxidation (Niki et al., 1993). The most commonly used form of VE in the poultry industry is DL-α-tocopherol acetate, which has high biological efficiency in preventing tissue oxidative damage (Hosomi et al., 1997; Panda and Cherian, 2014; Niki, 2016). Previous studies have shown that VE supplementation (200 IU/kg) early posthatch reduced WB severity in broilers at 58 days of age both phenotypically (Chapter 2) and microscopically (Chapter 3). Additionally, ALA is a short chain fatty acid with anti-inflammatory effects by inhibiting the release of pro-inflammatory cytokines such as tumor necrosis factor alpha (Li et al., 2014). Both antioxidant and anti-inflammatory properties make ALA a good candidate to reduce the WB severity. El-Senousey et al. (2013) showed that the optimal concentration of ALA for broilers was 400 to 800 mg/kg. Combining ALA and VE has been shown to function synergistically through the enhancement of antioxidant activity (Gonzalez-Perez and Gonzalez-Castaneda, 2006). After being reduced into dihydrolipoic acid, ALA helps recycle VE from its oxidized form (Sohaib et al., 2018). Therefore, VE and ALA both independently and in combination are highly likely to

influence the onset and development of WB myopathy through anti-oxidative and anti-inflammatory effects during the early posthatch period.

Although Chapter 2 showed that VE reduced the severity of WB at 58 days of age in broilers, there is no information about how VE and ALA combined may influence the onset and development of WB and when the beneficial changes will be initiated. Therefore, the objective of the current study was to evaluate the effects of VE, ALA, and combination of both on the onset, severity, and progression of the WB myopathy during the early posthatch period. The effects were determined based on the developmental changes in p. major muscle morphological structure and expression of genes related with muscle formation and growth, adipogenesis, extracellular matrix, oxidative stress and inflammation at 1 to 3 weeks of age in commercial broilers.

5.2 Materials and Methods

5.2.1 Birds and Experimental Diets

All bird protocols were approved by the Institutional Animal Care and Use Committee of The Ohio State University. A total of 160 newly hatched commercial Ross 708 broiler chicks were individually weighed, wing banded, and placed into pens immediately after hatch. Chicks were randomly divided into four groups, including a control group (corn-soybean meal basal diet), VE (160 mg/kg) supplemented group, ALA (500 mg/kg) supplemented group, and a combination of VE (160 mg/kg) and ALA (500 mg/kg) supplemented group. There were 10 pens per treatment, each pen included four

birds. Broilers had *ad libitum* access to feed and water. Diets were formulated to meet or exceed all NRC (National Research Council, 1994) nutritional requirements and recommendations in Aviagen's Ross broiler production handbook (Aviagen, 2016). Feed ingredients and calculated nutrient composition in the starter phase and grower phase are shown in Table 5.1 and Table 5.2, respectively.

5.2.2 Growth Performance

Broiler growth performance was evaluated every week from 1 to 3 weeks of age. Body weights were measured weekly throughout the trial. Average daily gain (ADG) was calculated as the rate of body weight gain per day. Feed intake (FI) was calculated by monitoring weekly feed disappearance. Feed conversion ratio (FCR) was calculated as the ratio of feed intake and body weight. Only one chick from the control group was dead at the beginning of the study and was not used for the growth performance calculation. At 1, 2 and 3 weeks, one chick from each pen was randomly selected and harvested. Final body weight was taken prior to exsanguination. The left p. major muscle was weighed and the right p. major muscle was used for p. major muscle morphological analysis and gene expression analysis. The p. major muscle weight was calculated as twice the weight of the left p. major muscle.

5.2.3 Phenotypic Wooden Breast Score

Phenotypic WB score was determined by palpation of the left p. major muscle. The evaluation was based on palpable firmness using a zero to three scale as described by Tijare et al. (2016). A score of zero represents no WB with the fillets being flexible in the entire

muscle. A score of one means mild WB with the fillets having firmness mainly in the anterior region. A score of two indicates moderate WB and the fillets are firm in the entire muscle except from mid to caudal region. A score of three represents severe WB and the fillets are extremely firm throughout the entire p. major muscle.

5.2.4 Pectoralis Major Muscle Morphology

The right p. major muscle was collected according to Velleman et al. (2003). Muscle fibers in the anterior portion of the muscle were dissected following the fiber orientation, tied to wooden applicator sticks to prevent contraction, fixed in 10% (vol/vol) buffered formalin (pH 7.0), and stored at 4 °C. Histological samples were dehydrated in a graded series of alcohols, cleared in Pro Par Clearant (Anatech, Battle Creek, MI) and paraffin embedded according to the procedure of Jarrold et al. (1999). Paraffin blocks were cross sectioned at 5 µm, mounted on Starfrost Adhesive slides (Mercedes Medical, Sarasota, FL), and hematoxylin and eosin stained as described by Jarrold et al. (1999). Each slide contained a minimum of four sections.

Digital photomicrographs were taken with an Olympus IX70 fluorescent microscope (Olympus America, Mellville, NY) and QImaging digital camera (QImaging, Burnaby, BC, Canada) equipped with CellSens Imaging software (Olympus America). Four photomicrographs from each section were taken at 100× and 200× magnification for p. major muscle morphology score and microscopic WB score evaluation. The p. major muscle morphology score was evaluated as described by Velleman et al. (2003). Samples were scored independently using a one to five scale by three trained panelists. A score of

one was given to samples with limited or no perimysial or endomysial connective tissue spacing, and excessive myofiber degradation. A score of five was given to samples with well-structured muscle fiber bundles and myofibers with ample perimysial and endomysial connective tissue spacing. Scores of two to four were intermediate. Microscopic WB score was identified based on the degree of fibrosis, necrosis, and immune cell infiltration, with a score of zero representing no necrosis, fibrosis, or immune cell infiltration, a score of one representing minimal necrosis, fibrosis, and immune cell infiltration, a score of two being intermediate, and a score of three representing severe necrosis, fibrosis, and immune cell infiltration.

Myofiber width, perimysial and endomysial width were measured from four photomicrographs per bird. At least 20 measurements per characteristic were taken in each photomicrograph at 200× magnification using Image Pro software (Media Cybernectics, Bethesda, MD).

5.2.5 RNA Extraction and Real-Time Quantitative PCR

Approximately a 0.5 g sample from the right p. major muscle was placed in RNAlater (Ambion, Grand Island, NY) for gene expression analysis from each bird after euthanasia. After 24 h, RNAlater was removed and samples were maintained at -20 ℃ until RNA extraction. Total RNA was extracted from muscle samples using RNAzol RT (Molecular Research Center, Madison, MI) according to manufacturer's protocol. Concentration of the total RNA was measured with a Nanodrop spectrophotometer ND-1000 (Thermo Fisher Scientific, Waltham, MA). The cDNA was synthesized with M-MLV

reverse transcriptase (Promega, Madison, WI) and real-time quantitative PCR (qPCR) was performed with the DyNAmo Hot Start SYBR Green qPCR kit (ThermoFisher, Waltham, MA) as described in Velleman et al. (2014). The reaction consisted of 1 µL of cDNA, 5 µL of DyNAmo Hot Start SYBR Green qPCR master mix, 0.5 µL of primer mixture (10 µM), and 3.5 µL of RNase-DNase free water. Genes whose expression is associated with muscle formation and growth, adipogenesis, extracellular matrix, oxidative stress and inflammation were selected as target sequences to be measured. Primer sequences of these genes and the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), are listed in Table 5.3. The qPCR was performed with appropriate cycling conditions as follows: denaturation (94°C for 15 min), amplification and quantification (35 cycles of 94°C for 30 s, 53°C for *SELE*; 55°C for *PPARγ*, *CEBPα*, and *GAPDH*; 58°C for *MyoD* and *MyoG*; and 60°C for *SDC4* for 30 s, and 72°C for 30 s), and final extension (72°C for 5 min). The PCR products were analyzed on a 1.0 % agarose gel to confirm amplification specificity. Gene expression was calculated as arbitrary units using the standard curve method, which was constructed for each target gene and housekeeping gene using serial dilutions of purified PCR products. Target gene expression was then normalized using *GAPDH* expression with each cDNA product concentration being divided by *GAPDH* concentration.

5.2.6 Statistical Analysis

Growth performance, p. major muscle morphology, and gene expression were analyzed as a completely randomized design using PROC MIXED procedure of SAS

version 9.4 software (SAS Institute INC., Cary, NC). Individual pen was identified as the experimental unit. Dietary treatments were used as a fixed effect. Least square means were estimated with the LSMEANS procedure and separated with the PDIFF option. Significance was accepted at $P \leq 0.05$.

5.3 Results

5.3.1 Growth Performance

Broiler growth performance in this study is shown in Table 5.4. Broilers with different dietary treatments did not have a significant difference in ADG, FCR, final body weight, or p. major weight at 1, 2, or 3 weeks of age ($P > 0.05$). However, broilers supplemented with VE and ALA independently had decreased FI at 3 weeks of age compared to the control group and the combination of VE and ALA group ($P < 0.01$).

5.3.2 Pectoralis Major Muscle Morphology

No phenotypic WB was identified by palpation from 1 to 3 weeks of age. However, mild WB with microscopic WB score of one was detected beginning at 1 week of age in all groups (Table 5.5). At 1 week of age, 50% of the p. major muscles were scored with mild microscopic WB and 50% had no WB in the control and ALA group. Broiler chicks in the VE group and combination of VE and ALA group had 60% mild WB and 40% no WB at 1 week of age. At 2 weeks of age, all of the dietary treatments had reduced WB severity microscopically compared to the control group. At this time in the control group, 60% of the p. major muscle samples exhibited a mild level of WB and 20% were moderate.

In contrast, no moderate WB cases were found in VE, ALA, and combination of VE and ALA groups at 2 weeks of age. In the VE and ALA group at 2 weeks of age, there was 50% mild WB and 50% had no WB. Combination of VE and ALA group had 70% no WB and 30% mild WB at 2 weeks of age. Wooden Breast severity was increased in the control group at 3 weeks of age with 10% no WB, 80% mild WB, and 10% moderate WB. The VE and combination of VE and ALA groups had 60% no WB and 40% mild WB at 3 weeks of age. The ALA group had 50% no WB and 50% mild WB at 3 weeks of age. Representative photomicrographs showing morphological structure of the p. major muscles affected or not affected with WB in the four dietary treatments at 3 weeks of age are shown in Figure 5.1. Fibrotic changes with fibrillar collagen replacing the myofibers were observed in the WB affected p. major muscle in all groups at 3 weeks of age (Figure 5.1A, 1C, 1E, 1F). Hypertrophic myofibers and fat cells were found in the WB affected tissues at 3 weeks of age as well. In contrast, the WB unaffected p. major muscles did not have these microscopic changes usually associated fibrosis (Figure 5.1B, D, F, H). Interestingly, there was no significant difference in fiber width, perimysial and endomysial connective tissue spacing, or morphology score among the dietary treatments within each age ($P > 0.05$; Table 5.6).

5.3.3 Pectoralis Major Muscle Gene Expression

In terms of genes associated with muscle formation and growth, *MyoD* expression was reduced by 6.6%, 9.0%, and 13.6% when diets were supplemented with VE and ALA independently or in combination at 3 weeks of age compared to the control group,

respectively ($P < 0.01$). Broilers fed dietary ALA and combination of VE and ALA had 37.9% and 65.1% decrease in *MyoG* expression at 3 weeks of age compared to the control group, respectively ($P < 0.01$). With regard to genes associated with adipogenesis, supplementation of VE and ALA independently or in combination had a 62.1%, 30.6%, and 51.6% reduction in *PPARγ* expression at 2 weeks of age, and a 50.0%, 285.7%, and 107.7% reduction at 3 weeks of age compared to the control group, respectively ($P < 0.01$). Vitamin E and ALA supplementation independently reduced *CEBPα* expression by 11.1% and 7.1% at 2 weeks of age compared to the control group, respectively ($P < 0.01$). Additionally, ALA and combination of VE and ALA group decreased *CEBPα* expression by 30.0% at 3 weeks of age compared to the control group ($P < 0.01$). Dietary ALA had a 13.0% and 20.0% reduction in *SELE*, an gene related with oxidative stress and inflammation, at 2 weeks ($P = 0.02$) and 3 weeks of age ($P < 0.01$) compared to the control group, respectively. In terms of gene related with extracellular matrix, there was no significant difference in *SDC4* expression among the dietary treatments within each age ($P > 0.05$).

5.4 Discussion

Prior morphological and genomic analyses have shown that severe oxidative stress (Mutryn et al., 2015; Abasht et al., 2016) and inflammation (Mudalal et al., 2015; Sihvo et al., 2017) are present in WB affected breast muscle. Oxidative stress and inflammation are thought to be caused by rapid posthatch growth (Kong et al., 2017). Since posthatch muscle

growth is dependent on satellite cells which are most active the first week after hatch (Mozdziak et al., 2002) and are sensitive to nutritional changes (Halevy et al., 2000; Velleman et al., 2010; Powell et al., 2014; Velleman et al., 2014), nutritional strategies to reduce oxidative stress and inflammation during the early posthatch period can likely be used to influence muscle growth and reduce the severity and incidence of myopathies like WB. Previous studies have identified that supplementation with the strong antioxidant VE during the starter phase (0 to 10 day) or grower phase (11 to 24 day) reduced the severity of WB (Chapter 2) and improved p. major muscle morphological structure (Chapter 3) at 58 days of age in broilers. Alpha lipoic acid augments immunity through anti-inflammatory activities and improves the antioxidant defense system (Ma et al., 2015). A combination of VE and ALA functions synergistically and will further increase antioxidant activity (Gonzalez-Perez and Gonzalez-Castaneda, 2006). Therefore, the present study investigated the effects of VE, ALA, and combination of both on the onset, severity, and progression of WB based on developmental changes in p. major muscle morphological structure and expression of genes associated with WB during the first 3 weeks posthatch.

In general, dietary VE and ALA did not have an impact on growth performance except for FI. Supplementation of VE and ALA independently decreased FI at 3 weeks of age. This is consistent with previous studies showing that VE (Konieczka et al., 2018) and ALA incorporation (Zhang et al., 2014) reduced growth performance. Zhang et al. (2014) contributed the impaired growth performance to the poor palatability due to the disulfide bond in ALA, which suppresses FI. El-Senousey et al. (2013) reported that 800 and 1200

mg/kg ALA resulted in decreased FI while 400 mg/kg ALA did not have influence on FI and FCR. This showed that different concentrations of ALA have varied effects on FI with higher level of ALA having a higher potential negative effect. However, combining VE and ALA ameliorated the negative influence on growth performance because of their synergistic effect on alleviating oxidative stress (Stilatha et al., 2010; Parveen et al., 2013), which was confirmed in the present study as well. Furthermore, dietary supplementation of VE along with ALA improved antioxidant potential and lipid stability of broiler breast meat (Sohaib et al., 2012).

Wooden Breast has been detected as early as 18 days of age by palpation in Ross 508 broiler chicks (Sihvo et al., 2017). Griffin et al. (2018) detected WB by palpation as early as 23 days in Ross 708 broiler chicks. In the current study with Ross 708 broiler chicks, there was no phenotypic detection of WB by 3 weeks of age using palpation. The differences in WB phenotypic identification by palpation are related to the differences in organization of the collagen between distinct genetic broiler lines (Velleman et al., 2017; Tonniges et al., 2019). Velleman et al. (2017) compared the collagen fibrils in three different genetic broiler lines. In a fast growing line with high frequency of WB phenotypic detection, tightly packed collagen fibrils were identified. In another fast growing line with low frequency of WB phenotypic detection, collagen fibrils were randomly aligned. In the slow growing line with no WB detected, collagen was sparsely distributed. The tightly packed collagen is associated with a high level of crosslink results in the hardness of p. major muscle. In contrast, the diffused collagen with a low level of crosslink does not

impact the toughness of the breast muscle. The nonreversible trivalent hydroxylysylpyridinium (HP) crosslink increases with age (Palokangas et al., 1992; Haus et al., 2007) and higher amounts of the HP crosslink result in less tender meat (McCormick, 1999; Purslow, 2018).

Microscopically, mild WB was observed beginning at 1 week of age in all groups. Supplemental VE and ALA altered the onset and development of microscopic WB in the present study. The control group had increasing WB severity microscopically from 1 to 3 weeks of age, while the VE group and combination of VE and ALA group did not show an increase in WB incidence from 1 to 3 weeks of age. Vitamin E and ALA supplementation independently and in combination decreased the WB severity at 2 and 3 weeks of age compared to the control group. This is consistent with previous studies that supplementation of VE (200 IU/kg) reduced the WB severity both by palpation (Chapter 2) and by microscopic evaluation (Chapter 3) in broilers at 58 days. Taken together, these data support that appropriate dietary supplementation early posthatch can positively affect long-term breast muscle development.

In terms of p. major muscle morphology, fiber width, perimysial, and endomysial connective tissue spacing were not influenced by the dietary VE and ALA supplementation both independently and in combination. Morphology score, which was evaluated based on perimysial and endomysial connective tissue spacing and myofiber structure, was not impacted. Similar findings were observed by Chapter 3 in broilers at 58 day.

In contrast, gene expression in the p. major muscle was significantly influenced by the dietary supplementation. Genes associated with muscle proliferation and differentiation, *MyoD* and *MyoG*, were differentially expressed in the dietary treatments at 3 weeks of age. Muscle growth after hatch is dependent on satellite cells (Moss and Leblond, 1971), which are most active the first week posthatch and then gradually become quiescent (Halevy et al., 2000). However, satellite cells can be reactivated to undergo regeneration process when the muscle is damaged (Schultz, 1989). They proliferate to increase myofiber number and differentiate by fusing with existing myofibers (Moss and Leblond, 1971). The MyoD and myogenic factor 5 transcriptional regulatory factors are necessary for proliferation (Rudnicki et al., 1993) while MyoG is required for differentiation leading to the formation of multinucleated myotubes (Hasty et al., 1993). The reduction in *MyoD* expression in VE and ALA group both independently and in combination, and reduced *MyoG* expression in ALA and combination of VE and ALA group compared to the control group at 3 weeks of age are suggestive of decreased proliferation and differentiation. Due to the activation of satellite cells with muscle fiber damage which occurs with WB, these data suggest that fewer muscle fibers were damaged and entered the regeneration process. The higher *MyoD* and *MyoG* expression along with the higher microscopic WB severity in the control group compared to the other dietary treatments at 3 weeks of age are in agreement with Velleman and Clark (2015) that expression of genes related with myogenic proliferation and differentiation are increased in the WB affected breast muscle.

Expression of *SELE*, which is related with oxidative stress and inflammation, was decreased at 2 and 3 weeks of age when the diets were supplemented with ALA compared to the control group. As an important adhesion molecule, SELE plays a key role in chronic and acute inflammation (Lundberg, 2000; Ley, 2003) and helps mediate leukocyte migration to inflammatory sites through leukocyte-endothelial interactions (Fries et al., 1993; Lucass et al., 1994). When there are oxidative signals involved in endothelium or intracellular oxidative damage, extracellular inducers such as cytokines are recruited, and nuclear factor-κB is further activated initiating a cascade of kinases and regulating *SELE* expression (Collins, 1993; Collins et al., 1995; Ghosh et al., 1998). In the current study, ALA supplementation reduced *SELE* expression at 2 and 3 weeks of age. This may also explain the reduced severity of WB at 2 and 3 weeks of age in the broiler chicks supplemented with ALA as *SELE* expression was decreased suggesting a lower level of oxidative stress and inflammation in the breast muscle.

Recent studies have shown that dysregulation of lipid deposition is closely associated with WB (Abasht et al., 2016; Zambonelli et al., 2017; Papah et al., 2017, 2018; Lake et al., 2019). Thus, genes involved in lipid metabolism such as *PPARγ* and *CEBPα* are important markers for the WB development. Both *PPARγ* and *CEBPα* are adipogenic genes mediating differentiation of muscle cells into adipocytes (Hu et al., 1995) and fatty acids metabolism (Kliewer et al., 1997; Rosen et al., 1999). The *PPARγ* is expressed early in adipogenesis leading to an up-regulation in the expression of *CEBPα* (Tontonoz et al., 1994; Yeh et al., 1995). Higher expression of *PPARγ* and *CEBPα* is commonly related with

increased intramuscular lipid deposition (Liu et al., 2017; Cui et al., 2018). In the present study, broilers supplemented with VE and ALA individually or in combination had decreased *PPARγ* and *CEBPα* expression, suggesting reduced fat deposition and WB severity.

In terms of gene related with extracellular matrix, there was no significant effect in *SDC4* expression among the treatments within each age. As a transmembrane heparan sulfate proteoglycan, SDC4 is associated with focal adhesion formation and cell migration (Longley et al., 1999; Couchman, 2003). The SDC4 activates protein kinase C alpha through the *SDC4* cytoplasmic domain (Woods and Couchman, 1992; Lee et al., 1998; Lim et al., 2003; Shin et al., 2013) and then activates Ras homolog family member A (RhoA) to modulate focal adhesion, cell migration, and proliferation (Woods et al., 2000; Dovas et al., 2006). No effect in *SDC4* expression indicates that there was no effect of the dietary supplementation on SDC4 mediated pathway of RhoA mediated cell migration.

In conclusion, administration of VE and ALA independently and in combination during the first 3 weeks post hatch may mitigate the onset and development of WB. Genes involved in muscle growth and development, adipogenesis, and oxidative stress and inflammation were differentially expression in broiler chicks supplemented with VE and ALA independently and in combination at 2 and 3 weeks of age, suggesting reduced p. major muscle degeneration and less dysregulated lipid deposition. The findings in the current study showed that VE and ALA may be used to reduce the incidence of WB as early as 2 weeks of age, which may be used as a tool to reduce WB incidence and severity

in broilers grown to market age. Future study should determine the effects of VE and ALA on the developmental onset, severity, and progression of WB of the broilers to an older age.

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Table 5.1 Feed ingredients and calculated nutritional composition of starter diets[1]

Item	Control	VE	ALA	VE and ALA
Ingredients, % as-fed				
Corn	51.40	51.39	51.35	51.34
Soybean meal	33.62	33.62	33.62	33.62
Poultry byproduct meal	7.50	7.50	7.50	7.50
Sodium chloride	0.22	0.22	0.22	0.22
Limestone	1.10	1.10	1.10	1.10
Dicalcium phosphate	0.46	0.46	0.46	0.46
Premix[2]	0.35	0.35	0.35	0.35
$_{L}$-Lys HCL	0.15	0.15	0.15	0.15
$_{DL}$-Met	0.34	0.34	0.34	0.34
$_{L}$-Thr	0.11	0.11	0.11	0.11
$NaHCO_3$	0.10	0.10	0.10	0.10
Selenium	0.10	0.10	0.10	0.10
Amprolium	1.000	1.00	1.00	1.00
Dl-α-tocopherol acetate	-	0.016	-	0.016
Soy oil	3.55	3.55	3.55	3.55
ALA	-	-	0.05	0.05
Calculated Nutrients and energy				
AME, kcal/kg	3000	3000	2999	2998
Protein, %	23.73	23.73	23.73	23.72
Calcium, %	0.96	0.96	0.96	0.96
Available phosphorus, %	0.48	0.48	0.48	0.48
Digestible Lys, %	1.28	1.28	1.28	1.28
Digestible Met+Cys, %	0.95	0.95	0.95	0.95

[1]Broilers in the control group were fed corn-soybean meal basal diet. Vitamin E (VE; 160mg/kg) and alpha lipoic acid (ALA; 500 mg/kg) were supplemented independently and in combination in the dietary treatments during the starter phase (0 to 7 day).
[2]The premix provides the following per kg of diet: vitamin A, 1,715,000 IU; vitamin D3, 6,000 IU; vitamin E, 12,600 IU; vitamin B12, 11 µg; folic acid, 1.5 mg; biotin, 150 µg; calcium carbonate, 25 mg; Mn, 60 mg; Zn, 40 mg; I, 0.33 mg; Fe, 80 mg; Cu, 8 mg; Se, 0.15 mg; and ethoxyquin, 150 mg (Provimi North America, Brookville, OH).

Table 5.2 Feed ingredients and calculated nutritional composition of grower diets[1]

Item	Control	VE	ALA	VE and ALA
Ingredients, % as-fed				
Corn	56.56	56.54	56.51	56.49
Soybean meal	28.06	28.06	28.06	28.06
Poultry byproduct meal	7.50	7.50	7.50	7.50
Sodium chloride	0.23	0.23	0.23	0.23
Limestone	1.02	1.02	1.02	1.02
Dicalcium phosphate	0.29	0.29	0.29	0.29
Premix[2]	0.35	0.35	0.35	0.35
$_{L}$-Lys HCL	0.15	0.15	0.15	0.15
$_{DL}$-Met	0.30	0.30	0.30	0.30
$_{L}$-Thr	0.09	0.09	0.09	0.09
$NaHCO_3$	0.10	0.10	0.10	0.10
Selenium	0.10	0.10	0.10	0.10
Amprolium	1.00	1.00	1.00	1.00
Dl-α-tocopherol acetate	-	0.016	-	0.016
Soy oil	4.25	4.25	4.25	4.25
ALA	-	-	0.05	0.05
Calculated Nutrients and energy				
AME, kcal/kg	3102	3102	3100	3100
Protein, %	21.56	21.56	21.56	21.56
Calcium, %	0.87	0.87	0.87	0.87
Available phosphorus, %	0.44	0.44	0.44	0.44
Digestible Lys, %	1.15	1.15	1.15	1.15
Digestible Met+Cys, %	0.87	0.87	0.87	0.87

[1]Broilers in the control group were fed corn-soybean meal basal diet. Vitamin E (VE; 160mg/kg) and alpha lipoic acid (ALA; 500 mg/kg) were supplemented independently and in combination in the dietary treatments during the grower phase (8 to 21 day).
[2]The premix provides the following per kg of diet: vitamin A, 1,715,000 IU; vitamin D3, 6,000 IU; vitamin E, 12,600 IU; vitamin B12, 11 µg; folic acid, 1.5 mg; biotin, 150 µg; calcium carbonate, 25 mg; Mn, 60 mg; Zn, 40 mg; I, 0.33 mg; Fe, 80 mg; Cu, 8 mg; Se, 0.15 mg; and ethoxyquin, 150 mg (Provimi North America, Brookville, OH).

Table 5.3 Primer sequences for real-time quantitative PCR

Gene	Accession number	Forward primer (5’ to 3’)	Reverse primer (5’ to 3’)	Amplicon (bp) size
Muscle formation and growth				
MyoD[1]	AY641567.1	GACGGCATGAT GGAGTACAG	AGCTTCAGCTG GAGGCAGTA	234
MyoG[2]	AY560111.3	CCTTTCCCACTC CTCTCCAAA	GACCTTGGTCG AAGAGCAACT	201
Adipogenesis				
PPARγ[3]	NM_001001 460.1	CCACTGCAGGA ACAGAACAA	CTCCCGTGTCA TGAATCCTT	249
CEBPα[4]	NM_001031 459.1	CAGTGGACAAG AACAGCAACGA	CCTTCACCAGC GAGCTTTCG	227
Extracellular matrix				
SDC4[5]	NM_001007 869.1	CCAACAGCAGC ATCTTTGAA	GATGGGTTTCT TCCCAAGGT	155
Oxidative stress and inflammation				
SELE[6]	XM_025153 162	CAACAGAGAGC AGTCGCTGA	GTAAGCCCGTA AGCAAGAAGA G	157
Housekeeping gene				
GAPDH[7]	U94327.1	GAGGGTAGTGA AGGCTGCTG	CCACAACACGG TTGCTGTAT	175

[1]*MyoD* = Myogenic determination factor 1.
[2]*MyoG* = Myogenin.
[3]*PPARγ* = Peroxisome proliferator-activated receptor gamma.
[4]*CEBPα* = CCAAT/enhancer-binding protein alpha.
[5]*SDC4* = Syndecan-4.
[6]*SELE* = Selectin E.
[7]*GAPDH* = Glyceraldehyde-3-phosphate dehydrogenase.

Table 5.4 Effect of vitamin E and alpha lipoic acid on broiler growth performance from 1 to 3 weeks posthatch

Item	Age (week)	Treatments[1]				SEM[2]	*P*-Value
		Control	VE	ALA	VE and ALA		
ADG[3] (g)	1	13.52	13.54	13.36	13.36	0.28	0.95
	2	25.47	25.55	25.74	25.59	0.65	0.99
	3	29.59	29.35	29.84	30.43	0.80	0.82
FI[4] (g)	1	14.49	14.73	14.12	14.73	0.18	0.06
	2	38.93	39.43	38.42	39.46	0.35	0.13
	3	52.52[a]	50.00[b]	50.34[b]	52.94[a]	0.47	< 0.01
FCR[5]	1	1.06	1.10	1.06	1.11	0.03	0.21
	2	1.53	1.58	1.53	1.57	0.04	0.79
	3	1.80	1.72	1.73	1.78	0.04	0.52
Final body weight (g)	1	143.03	138.96	136.51	147.09	4.04	0.28
	2	338.56	362.76	346.71	342.61	11.75	0.50
	3	507.72	498.7	517.11	507.19	12.99	0.79
P. major[6] weight (g)	1	10.30	9.70	8.56	10.14	0.50	0.09
	2	27.86	31.98	31.14	30.46	1.94	0.51
	3	62.22	57.76	61.18	62.66	2.36	0.46

[a-b]Values within a row without a common letter are significantly different ($P \leq 0.05$).
[1]Values are means of 10 pens per treatment, each pen included three birds. Broilers in the control group were fed corn-soybean meal basal diet. Vitamin E (VE; 160mg/kg) and alpha lipoic acid (ALA; 500 mg/kg) were supplemented independently and in combination in the dietary treatments during the entire study (1 to 3 weeks).
[2]SEM = Standard error of means.
[3]ADG = Average daily gain.
[4]FI = Feed intake.
[5]FCR = Feed conversion ratio.
[6]P. major = Pectoralis major muscle.

Table 5.5 Effect of vitamin E and alpha lipoic acid on microscopic Wooden Breast (WB) score of broiler pectoralis major muscle

Age (week)	WB score[3]	Treatments[1], n (%)[2]			
		Control	VE	ALA	VE and ALA
1	0	5 (50)	6 (60)	5 (50)	6 (60)
	1	5 (50)	4 (40)	5 (50)	4 (40)
	2	0	0	0	0
	3	0	0	0	0
2	0	2 (20)	5 (50)	5 (50)	7 (70)
	1	6 (60)	5 (50)	5 (50)	3 (30)
	2	2 (20)	0	0	0
	3	0	0	0	0
3	0	1 (10)	6 (60)	5 (50)	6 (60)
	1	8 (80)	4 (40)	5 (50)	4 (40)
	2	1 (10)	0	0	0
	3	0	0	0	0

[1]Broilers in the control group were fed corn-soybean meal basal diet. Vitamin E (VE; 160mg/kg) and alpha lipoic acid (ALA; 500 mg/kg) were supplemented independently and in combination in the dietary treatments during the entire study (1 to 3 weeks).
[2]The number (percentage) of the broilers with different WB score at each age within each dietary treatments.
[3]The WB scores were based on the degree of fibrosis, necrosis, and immune cell infiltration. Score zero = none WB, one = mild WB, two = moderate WB, three = severe WB.

Table 5.6 Effect of vitamin E and alpha lipoic acid on fiber width, perimysial and endomysial connective tissue spacing, and morphology score of broiler pectoralis major muscle

Item	Age (week)	Treatments[1]				SEM[2]	*P*-Value
		Control	VE	ALA	VE and ALA		
Fiber width (μm)	1	5.39	5.29	5.14	5.05	0.16	0.55
	2	8.35	8.98	9.07	9.58	0.36	0.15
	3	9.72	10.09	10.61	10.39	0.38	0.73
Perimysium (μm)	1	9.07	9.17	8.99	8.86	0.30	0.92
	2	8.48	8.74	8.57	8.65	0.42	0.98
	3	7.51	7.47	7.49	7.77	0.34	0.84
Endomysium (μm)	1	2.39	2.48	2.30	2.62	0.13	0.36
	2	2.31	2.20	2.28	2.04	0.09	0.20
	3	2.68	2.60	2.65	2.65	0.14	0.94
Morphology score[3]	1	1.87	2.29	2.05	2.27	0.13	0.14
	2	2.14	2.48	2.51	2.23	0.14	0.18
	3	2.65	2.90	3.04	2.61	0.18	0.51

[1]Values are means of 10 pens per treatment, each pen included three birds. Broilers in the control group were fed corn-soybean meal basal diet. Vitamin E (VE; 160mg/kg) and alpha lipoic acid (ALA; 500 mg/kg) were supplemented independently and in combination in the dietary treatments during the entire study (1 to 3 weeks).
[2]SEM=Standard error of means.
[3]Morphology score was evaluated using a one to five scale by three trained panelists. A score of one was given to samples with limited or no perimysial or endomysial connective tissue spacing, and excessive myofiber degradation and necrosis. A score of five was given to samples with well-structured muscle fiber bundles and myofibers with ample perimysial and endomysial connective tissue spacing. Scores of two to four were intermediate.

Table 5.7 Effect of vitamin E and alpha lipoic acid on gene expression in broiler pectoralis major muscle

Item (arbitrary unit)	Age (week)	Treatments[1]				SEM[2]	*P*-value
		Control	VE	ALA	VE and ALA		
Muscle formation and growth							
MyoD[3]	1	16.30	15.02	16.24	16.51	0.61	0.29
	2	15.80	14.38	15.12	14.39	0.58	0.45
	3	11.26^{a}	10.56^{b}	10.33^{bc}	9.91^{c}	0.32	< 0.01
MyoG[4]	1	0.79	0.66	0.73	0.79	0.07	0.47
	2	2.21	2.12	2.02	2.17	0.10	0.66
	3	1.42^{a}	1.26^{ab}	1.03^{bc}	0.86^{c}	0.09	< 0.01
Adipogenesis							
PPARγ[5]	1	0.049	0.039	0.051	0.048	0.006	0.36
	2	0.047^{a}	0.029^{b}	0.036^{b}	0.031^{b}	0.003	< 0.01
	3	0.027^{a}	0.018^{b}	0.007^{c}	0.013^{bc}	0.002	< 0.01
CEBPα[6]	1	0.030	0.025	0.030	0.025	0.002	0.31
	2	0.030^{a}	0.027^{b}	0.028^{b}	0.029^{a}	0.001	< 0.01
	3	0.013^{a}	0.014^{a}	0.010^{b}	0.010^{b}	0.001	< 0.01
Extracellular matrix							
SDC4[7]	1	0.039	0.036	0.028	0.043	0.009	0.71
	2	0.098	0.084	0.095	0.110	0.013	0.72
	3	0.180	0.130	0.180	0.130	0.030	0.43
Oxidative stress and inflammation							
SELE[8]	1	0.048	0.053	0.042	0.039	0.007	0.29
	2	0.061^{a}	0.064^{a}	0.054^{b}	0.058^{ab}	0.002	0.02
	3	0.009^{a}	0.009^{a}	0.005^{b}	0.010^{a}	0.001	< 0.01

$^{a-c}$Means within a row without a common letter are significantly different ($P \leq 0.05$).
[1]Values are means of 10 pens per treatment, each pen included three birds. Broilers in the control group were fed corn-soybean meal basal diet. Vitamin E (VE; 160mg/kg) and alpha lipoic acid (ALA; 500 mg/kg) were supplemented independently and in combination in the dietary treatments during the entire study (1 to 3 weeks).
[2]SEM = Standard error of means; [3]*MyoD* = Myogenic determination factor 1.
[4]*MyoG* = Myogenin; [5]*PPARγ* = Peroxisome proliferator-activated receptor gamma.
[6]*CEBPα* = CCAAT/enhancer-binding protein alpha.
[7]*SDC4* = Syndecan-4; [8]*SELE* = Selectin E.

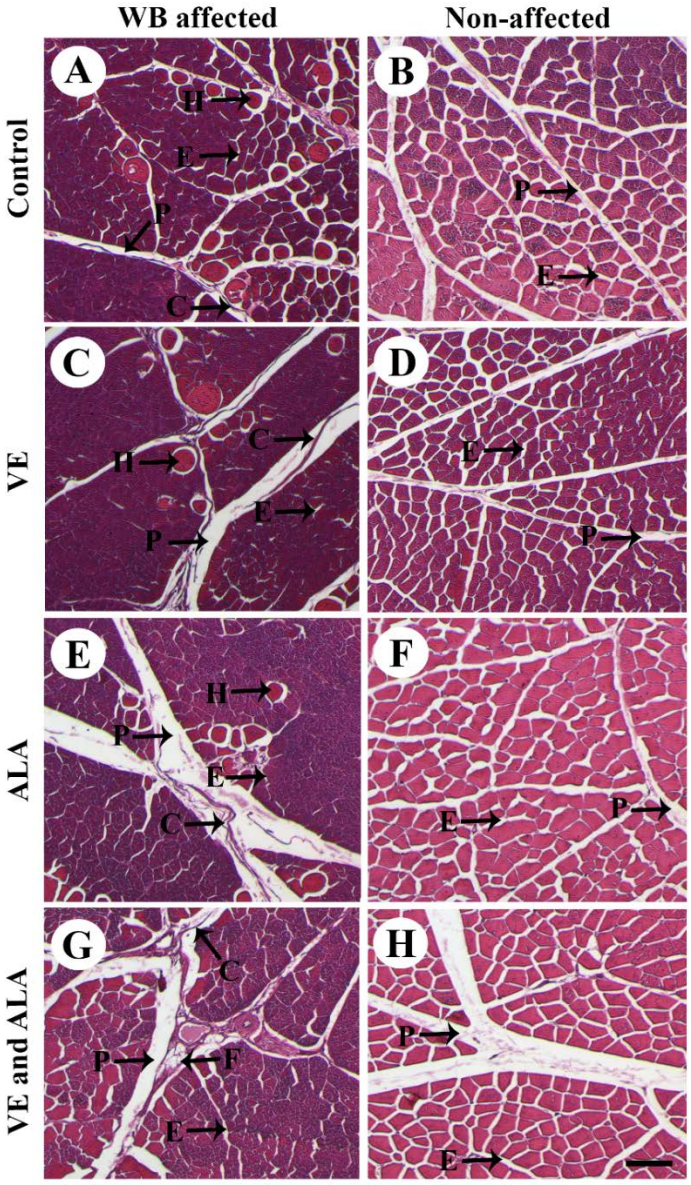

Figure 5.1 Representative photomicrographs of Wooden Breast (WB) affected (A, C, E, G) or not affected (B, D, F, H) pectoralis major muscle samples in the four treatments at 3 weeks of age. Broilers in the control group (A, B) were fed corn-soybean meal basal diet. Vitamin E (VE; 160mg/kg) was supplemented in the VE group (C, D) and alpha lipoic acid (ALA; 500 mg/kg) was supplemented in the ALA group (E, F). Combination of VE and ALA was supplemented in the VE and ALA group (G, H). C = Collagen; F: Fat cell; H: Hypertrophic myofiber; P = Perimysial connective tissue; E = Endomysial connective tissue. Scale bar = 100 μm.

Chapter 6: Effect of vitamin E and alpha lipoic acid on intestinal development associated with Wooden Breast myopathy in broilers

Abstract

Intestinal development is closely associated with the inflammatory Wooden Breast (WB) myopathy. Vitamin E (VE) and alpha lipoic acid (ALA) with antioxidant and anti-inflammatory effects were used independently and in combination to evaluate their effects on intestinal developmental changes in ileal morphology and expression of genes related with gut nutrient transport, barrier integrity, and inflammation in broilers during the first 3 weeks posthatch. A total of 160 newly hatched Ross 708 broiler chicks were randomly assigned into a control and three dietary treatments with 10 replicates of four birds each. Supplementation of VE (160 mg/kg) and ALA (500 mg/kg) independently and in combination were fed during the first 3 weeks. At 1, 2 and 3 weeks of age, one chick from each pen was harvested. Plasma VE concentration and ileal morphology were determined. Gene expression was measured by real-time quantitative PCR. Broilers in VE and combination of ALA and VE group had higher plasma VE concentration than the control and ALA group at 1, 2, and 3 weeks of age ($P < 0.01$). All dietary treatments increased ileal villus height at 1 wk of age ($P < 0.01$) and decreased intraepithelial lymphocytes at 3 weeks of age compared to the control ($P \leqslant 0.05$). Combination of VE and ALA increased collagen type IV alpha 1 chain expression ($P \leqslant 0.05$) and improved basement membrane structure indicating increased gut barrier integrity at 2 and 3 weeks of age compared to the

control. Expression of *lipopolysaccharide-induced tumor necrosis factor-alpha factor* associated with inflammation was decreased in all dietary treatments at 3 weeks of age compared to the control ($P < 0.01$). Ileal morphology and gene expression were closely correlated with breast muscle morphology and gene expression. These results suggest that VE and ALA especially when they were combined in the diet had positive effects on mitigating intestinal inflammation and improving nutrient transport beginning at 1 week of age, which is likely critical in reducing the severity of WB.

6.1 Introduction

Improvements in breast meat production associated with fast-growing heavy weight broilers has resulted in breast muscle myopathies like Wooden Breast (WB) (Brewer et al., 2012; Sihvo et al., 2014; Tijare et al., 2016; Mazzoni et al., 2020). Wooden Breast has been identified globally (Kuttappan et al., 2017; Xing et al., 2019; Hasegawa et al., 2020) creating considerable economic losses because of unacceptable meat quality and product downgrades (Sihvo et al., 2014; Kuttappan et al., 2016). Wooden Breast is phenotypically characterized with a rigid pectoralis major (p. major: breast muscle) muscle upon palpation (Sihvo et al., 2014). Histologically, WB affected breast muscle has impaired morphological structure with moderate or severe myodegeneration such as myofiber necrosis (Sihvo et al., 2014; Clark and Velleman, 2017), fibrosis (Velleman and Clark, 2015; Soglia et al., 2016), and inflammatory cell accumulation (Soglia et al., 2016; Sihvo et al., 2017). Breast muscle affected with WB have severe inflammation (Mutryn et

al., 2015; Zambonelli et al., 2017), oxidative stress (Mutryn et al., 2015; Abasht et al., 2016; Brothers et al., 2019), and dysregulated lipid metabolism (Abasht et al., 2019; Brothers et al., 2019; Lake and Abasht, 2020).

Gastrointestinal growth and development are closely associated with inflammatory WB myopathy because inflammation is often a systemic process affecting various physiological systems in the entire body (Bourikas and Papadakis, 2009; Chawla, 2011). Systemic inflammation can be produced when the gastrointestinal structure and function are negatively altered, contributing to inflammation throughout the entire body including breast muscle (Mafra et al., 2014). Chapter 4 has found that the p. major muscle morphology and expression of genes related with inflammation were closely correlated with intestinal inflammation. Intestinal development during the early posthatch period affects growth performance, breast muscle morphology (Noy and Sklan, 1998), and inflammation (Dibner et al., 1998) in broiler chicks. In addition, the small intestinal structure and function are sensitive to nutrition early posthatch with intestinal maturation being influenced by nutritional changes (Geyra et al., 2001; Mahmoud and Edens, 2012). Therefore, early posthatch nutritional interventions to reduce intestinal inflammation and oxidative stress will likely influence intestinal development as well as WB development.

Alpha lipoic acid (ALA) is a short chain fatty acid with anti-inflammatory properties through inhibiting the release of pro-inflammatory cytokines such as tumor necrosis factor (TNF) alpha and interleukin 6 (Li et al., 2014; Ma et al., 2015). Moreover, ALA has antioxidant effects by scavenging the free radicals thereby reducing oxidative

stress (El-Senousey et al., 2013). The antioxidant and anti-inflammatory effects will make ALA better to enhance immunity while improving the antioxidant defense system (Ma et al., 2015). Vitamin E (VE) is a powerful antioxidant which can prevent tissue oxidative damage (Niki et al., 1993). DL-α-tocopherol acetate is a commonly used form of VE in the poultry industry (Voljč et al., 2011; Panda and Cherian, 2014). Studies have shown that VE supplementation early posthatch not only reduced WB severity (Chapter 2 and 3) but also improved intestinal structure and mitigated intestinal inflammation in broilers at 58 day of age (Chapter 4). Combination of VE and ALA has a synergistic function to enhance antioxidant activity (Gonzalez-Perez and Gonzalez-Castaneda, 2006).

Chapter 5 found that dietary VE and ALA supplemented independently and in combination had positive effects on mitigating WB severity as early as 2 weeks of age. This indicates that the beneficial changes in p. major muscle morphology as shown by Chapter 3 in broilers at market age was probably initiated early posthatch. To identify if the intestinal changes followed a similar timeline as that observed for p. major muscle development, VE and ALA independently and in combination were used to supplement commercial broiler diets immediately after hatch to determine their effects on intestinal development. The intestinal developmental effect was evaluated based on developmental changes in ileal morphological structure and expression of genes related with gut nutrient transport, barrier integrity, and inflammation at 1 to 3 weeks of age in commercial broilers.

6.2 Materials and Methods

6.2.1 Birds and Experimental Diets

All bird protocols were approved by the Institutional Animal Care and Use Committee of The Ohio State University. A total of 160 newly hatched commercial Ross 708 broiler chicks were individually weighed, wing banded, and placed into pens immediately after hatch. Chicks were randomly divided into four groups, including a control group (corn-soybean meal basal diet), VE (160 mg/kg) supplementation group, ALA (500 mg/kg) supplementation group, and a combination of VE (160 mg/kg) and ALA (500 mg/kg) supplementation group. There were 10 pens per treatment, each pen included four birds. Broilers had *ad libitum* access to feed and water. Diets were formulated to meet or exceed all NRC (National Research Council, 1994) nutritional requirements and recommendations in Aviagen's Ross broiler production handbook (Aviagen, 2016). Feed ingredients and calculated nutrient composition in the starter phase and grower phase are shown in Chapter 5. At 1, 2 and 3 weeks of age, one chick from each pen was harvested in accordance with humane and commercial slaughter procedures.

6.2.2 Plasma α-Tocopherol Measurement

Approximately 1 mL of whole blood was collected from each bird after euthanasia. To allow the blood to clot, the blood samples were then placed at room temperature for around 30 min. Plasma was isolated from the coagulated blood by centrifugation (1500g, 15 min 4 °C) and stored at - 80 °C for further analysis. Plasma α-tocopherol was measured as described by Traber et al. (2017). Briefly, 100 µL of plasma sample was mixed with 1%

ascorbic acid and β-glucuronidase, incubated at 37 °C for 1 h, and cooled to room temperature. Four mL diethyl ether was used to extract the plasma, and an aliquot of the ether fraction was collected and dried under nitrogen. The samples were subsequently resuspended in 1:1 (vol:vol) water:methanol for injection into the liquid chromatography-tandem mass spectrometer (API3000; Sciex, Ontario, Canada) equipped with a turbo ion-spray source that was set to negative mode. The plasma α-tocopherol concentration was calculated from the standard curve that was generated from peak areas of authentic non deuterated compounds.

6.2.3 Intestinal Morphology

To evaluate intestinal morphology, a 3 cm long section of the ileum was obtained from each broiler. Tissue samples were immediately fixed in 10% (vol/vol) buffered formalin (pH 7.0) and stored at room temperature. Histological samples were dehydrated in a graded series of alcohols, cleared in Pro Par Clearant (Anatech, Battle Creek, MI), and paraffin embedded according to the procedure of (Jarrold et al., 1999). Paraffin blocks were cross sectioned at 5 µm and mounted on Starfrost Adhesive slides (Mercedes Medical, Sarasota, FL). The slides were either hematoxylin and eosin stained for measurements of villus height, crypt depth, villus width, distance between villi, and counting of intraepithelial lymphocytes (IELs) and epithelial cells number in villi or Periodic Acid Schiff (PAS; Thermo Fisher Scientific, Waltham, MA) stained for goblet cell counting and basement membrane structure evaluation. The hematoxylin and eosin staining was followed the method as described by Velleman et al. (2002). After deparaffinization and

rehydration, the slides were rinsed with hematoxylin for 4 min, running tap water for 10 min, and eosin Y for 2 min, dehydrated and mounted. In terms of the PAS staining, the slides were deparaffinized, rehydrated, incubated in periodic acid for 5 min, washed and incubated with Schiff's reagent for 15 min, and washed in running tap water for 10 min. After staining in hematoxylin for 1 min, slides were dehydrated and mounted. Each slide contained a minimum of four sections and imaged with a QImaging digital camera (QImaging, Burnaby, BC, Canada) attached to an Olympus IX 70 microscope (Olympus America, Mellville, NY).

At least four photomicrographs from each sample were taken for measurement. In hematoxylin and eosin stained slides, villus height was determined as the distance between the tip of the villi and the villus crypt junction. Crypt depth was measured as the distance of the invagination between two adjacent villi. Villus width was measured at the middle part of the villi. Distance between villi was determined as the distance between the adjacent villi at the base of the villi. Measurements were taken in 10 well-structured villi and crypts from each section of each sample using Image J 1.8.0 software (National Institutes of Health, Bethesda, MD). The ratio of villus height to crypt depth was calculated as the ratio of villus height and crypt depth. Surface area of the villi was calculated as $(2\pi) \times$ (villus width/2) × (villus height) (de Los Santos et al., 2007). Intraepithelial lymphocytes number and epithelial cells number in villi were determined. The IELs are small round cells with nucleus centrally located and with little cytoplasm inside (Wilson et al., 1986). Epithelial cells were counted to calculate the number of IELs per 100 epithelial cells. In PAS stained

slides, basement membrane structure was observed and appears as a dense sheet underlying the epithelial cells. Goblet cells were stained purple and were counted as the number of goblet cell per 100 µm of the villi.

6.2.4 RNA Extraction and Real-Time Quantitative PCR

Approximately 0.5 g ileal mucosal scraping was isolated from the ileum and stored at -80 °C until use. Total RNA was extracted from ileal mucosal scrapings using RNAzol RT (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol. Total RNA concentration was measured with a Nanodrop spectrophotometer ND-1000 (Thermo Fisher Scientific, Waltham, MA). The cDNA was synthesized with M-MLV reverse transcriptase (Promega, Madison, WI) and real-time quantitative PCR (qPCR) was done with DyNAmo Hot Start SYBR Green qPCR kit (ThermoFisher, Waltham, MA) as described in Velleman et al. (2014). The reaction contained 1 µL of cDNA, 5 µL of DyNAmo Hot Start SYBR Green qPCR master mix, 0.5 µL of primer mixture, and 3.5 µL of RNase-DNase free water. Solute carrier family 15 member 1 (*SLC15A1*), polypeptide N-acetylgalactosaminyltransferase 2 (*GALNT2*) associated with nutrient transport, mucin 2 (*MUC2*) and collagen type IV alpha 1 chain (*COL4A1*) associated with gut barrier integrity, and lipopolysaccharide-induced tumor necrosis factor-alpha factor (*LITAF*), and interferon gamma (*IFNG*) associated with inflammation were selected as target sequences to be measured. Primer sequences of these genes and the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), are listed in Table 6.1. The qPCR was performed with the following cycling conditions: denaturation (94°C for 15 min),

amplification and quantification (35 cycles of 94°C for 30 s, 53°C for *SLC15A1*, *GALNT2*, *MUC2*, and *COL4A1*; 55°C for *GAPDH*; and 60°C for *LITAF* and *IFNG* for 30 s, and 72°C for 30 s), and final extension (72°C for 5 min). The PCR products were sequenced for its amplification specificity in Molecular and Cellular Imaging Center, The Ohio State University, Wooster, OH. Gene expression was calculated as arbitrary units using the standard curve method, which was constructed for each target gene and housekeeping gene using serial dilutions of purified PCR products. Target gene expression was then normalized using *GAPDH* expression with each cDNA product concentration being divided by *GAPDH* concentration.

6.2.5 Statistical Analysis

Plasma VE, intestinal morphological attributes, and gene expression were analyzed as a completely randomized design using PROC MIXED procedure of SAS version 9.4 software (SAS Institute INC., Cary, NC). Individual pen was identified as the experimental unit. Dietary treatments were used as a fixed effect. Least square means were estimated with the LSMEANS procedure and separated with the PDIFF option. Significance was accepted at $P \leq 0.05$. To analyze correlation between gut development and WB development, final body weight, p. major muscle weight, and p. major muscle morphology and gene expression from Chapter 5 were used for correlation coefficients analysis with ileal morphology and gene expression. Pearson correlation coefficients were determined with the CORR procedure of SAS. The $P \leq 0.05$ was considered as a significant difference.

6.3 Results

6.3.1 Plasma α-Tocopherol Concentration

Plasma α-tocopherol concentration in the broilers used in this study are shown in Table 6.2. Broilers in the VE group and combination of ALA and VE group had higher plasma α-tocopherol concentration than the control and ALA group at 1, 2, and 3 weeks of age ($P < 0.01$). Broilers supplemented with a combination of VE and ALA had 20.33% increased concentration of plasma α-tocopherol compared to those supplemented with VE independently at 3 weeks of age ($P = 0.04$).

6.3.2 Ileal Morphology

Broiler ileal morphology at 1 to 3 weeks of age are shown in Table 6.3. Villus height was increased by 4.74%, 12.62%, and 8.13% in VE, ALA, and combination of VE and ALA groups compared to the control group at 1 week of age, respectively ($P < 0.01$). There was a 2.64% increase of villus height in the combination of VE and ALA group compared to the control group at 2 weeks of age ($P = 0.02$), and a 3.94% and 3.74% increase of villus height in the ALA ($P < 0.01$) and combination of VE and ALA groups ($P = 0.01$) compared to the control group at 3 weeks of age. Broilers in the ALA group and combination of VE and ALA group had a 16.08% and 12.04% decrease in the number of IELs compared to the control group at 2 weeks of age ($P < 0.01$). Supplementation of VE ($P = 0.03$), ALA ($P < 0.01$), and combination of VE and ALA ($P < 0.01$) decreased the number of IELs by 9.40%, 14.25%, and 15.50% compared to the control group at 3 weeks of age. There was no significant difference in crypt depth, the ratio of villus height to crypt

depth, villus width, surface area, distance between villi, or goblet cell number among the treatments within each age ($P > 0.05$). The integrity of the basement membrane was increased in the VE, ALA, and combination of VE and ALA groups, especially in the combination of VE and ALA group, at 2 weeks of age compared to the control group as shown in Figure 6.1. The basement membrane structure in the control group was less well defined with frequent fissures in the basement membrane compared to the other treatments.

6.3.3 Ileal Gene Expression

In terms of genes associated with nutrient transport, broilers fed dietary ALA ($P = 0.01$) and combination of VE and ALA ($P < 0.01$) had 6.89% and 8.14% increased *SLC15A1* expression at 2 weeks of age compared to the control group (Table 6.4). Supplementation of VE ($P = 0.04$), ALA ($P = 0.02$), and combination of VE and ALA ($P = 0.01$) had 5.05%, 5.53 %, and 6.00% increase in *SLC15A1* expression at 3 weeks of age compared to the control group, respectively. The VE ($P < 0.01$) and combination of VE and ALA ($P = 0.02$) supplementation increased 21.63% and 17.87% *GALNT2* expression at 2 weeks of age compared to the control group. Supplementation of ALA ($P < 0.01$) and combination of VE and ALA ($P = 0.02$) increased 13.33% and 10.86% *GALNT2* expression at 3 weeks of age compared to the control group. With regard to genes associated with gut barrier integrity, *MUC2* expression was increased by 16.19% and 9.46% when broilers were supplemented with VE ($P < 0.01$) and combination of VE and ALA ($P = 0.03$) at 3 weeks of age compared to the control group. Dietary VE, ALA, and combination of VE and ALA increased 6.80%, 5.77%, and 11.50% *COL4A1* expression at 2 weeks compared

to the control group ($P < 0.01$). Combination of VE and ALA supplementation increased 4.55% *COL4A1* expression at 3 weeks of age compared to the control group ($P = 0.01$). In terms of genes related with inflammation, expression of *LITAF* was decreased by 13.25%, 27.56%, and 32.19% in VE, ALA, and combination of VE and ALA groups at 3 weeks of age compared to the control group ($P < 0.01$). There was no significant difference in *IFNG* expression among the treatments within each age ($P > 0.05$).

6.3.4 Correlation Coefficient of Morphology and Gene Expression

Correlation coefficients between ileal morphology and gene expression, and broiler final body weight, p. major muscle weight, morphology and gene expression are shown in Table 6.5. Broiler final body weight and p. major muscle weight were positively correlated with ileal villus height, crypt depth, the ratio of villus height to crypt depth, surface area, and expression of *MUC2*, *SLC15A1*, and *GALNT2* ($P \leq 0.05$). The p. major muscle morphology score, with a higher score representing more well-structured muscle fiber, was positively correlated with ileal villus height, crypt depth, surface area, ileal expression of *MUC2* and *GALNT2* ($P \leq 0.05$). The p. major muscle fiber width was positively correlated with ileal villus height, crypt depth, the ratio of villus height to crypt depth, surface area, and expression of *MUC2* ($P < 0.01$). Expression of the lipid metabolism genes *PPARγ* and *SELE* which are associated with inflammation in the p. major muscle was negatively correlated with ileal villus height and the ratio of villus height to crypt depth ($P \leq 0.05$).

6.4 Discussion

Wooden Breast affected breast muscle is characterized by severe inflammation (Mutryn et al., 2015; Zambonelli et al., 2017) and oxidative stress (Mutryn et al., 2015; Abasht et al., 2016; Brothers et al., 2019). The development of WB is closely associated with intestinal structure and intestinal inflammatory state (Chapter 4). Previous studies have identified that supplementation with the antioxidant VE early posthatch reduced WB severity (Chapter 2 and 3) and reduced intestinal inflammation at market age in broilers (Pitargue et al., 2019; Chapter 4). The beneficial effects of VE and ALA with antioxidant and anti-inflammatory activities on mitigating the WB severity was initiated as early as 2 weeks of age of the broilers (Chapter 5). Therefore, effects of VE (160 IU/kg) and ALA (500 mg/kg) independently and in combination on developmental changes in intestinal morphology and expression of genes related with nutrient transport, barrier integrity, and inflammation at 1 to 3 weeks of age in commercial broilers were further investigated in the current study to identify the relationship between intestinal development and the onset of WB in the p. major muscle.

Intestinal structure is an essential attribute reflective of gut nutrient absorption (Celi et al., 2017). Villus height is an important morphological attribute for nutrient absorption and transport in the gastrointestinal system. It was increased in the VE and ALA dietary treatments both independently and in combination beginning at1 week of age compared to the control group, with the combination of VE and ALA group having the highest villus height. This is consistent with Yoo et al. (2016) that combination of VE and ALA increased

villus height in broilers. The digestive and absorptive capacity could be enhanced with an increased villus height through higher surface area of nutrient absorption and brush border enzymes expression (Yamauchi et al., 1996). The increased villus height in the dietary treatments is suggestive of an improvement in ileal morphology, nutrient uptake and absorptive efficiency beginning at1 week of age from the VE and ALA supplementation both independently and in combination. The combination of VE and ALA had a maximal beneficial effect.

Genes associated with gut nutrient transport were differentially expressed in the dietary treatments as well. The *SLC15A1* expression was increased in ALA and combination of VE and ALA groups at 2 weeks of age and in VE, ALA, and combination of VE and ALA groups at 3 weeks of age compared to the control group. This is in agreement with Chapter 4 that VE supplementation from 0 to 10 day posthatch increased ileal *SLC15A1* expression compared to the control at 58 days of age in broilers, which suggests that the effect of VE on *SLC15A1* expression in broilers at market age could be initiated as early as 2 weeks of age. The SLC15A1, also called peptide transporter 1, belongs to the superfamily proton oligopeptide transporters (Ingersoll et al., 2012). It is responsible for transportation of dipeptides and tripeptides in the enterocytes (Osmanyan et al., 2018), a major regulatory process of protein degradation and utilization (Gaildrat et al., 2005). The higher *SLC15A1* expression is suggestive of greater protein digestion and absorption in the broilers supplemented with VE and ALA independently and in combination compared to the control. Another gene related with nutrient transport,

GALNT2, had higher expression in VE and combination of VE and ALA groups at 2 weeks of age, and ALA and combination of VE and ALA groups at 3 weeks of age compared to the control. The *GALNT2* encodes polypeptide N-acetylgalactosaminyltransferase 2，which is a member of glycosyltransferase 2 protein family (Wu et al., 2011). The polypeptide N-acetylgalactosaminyltransferase 2 then activate mucin type O-glycosylation of peptides with N-acetyl galactosamine transferring to the hydroxyl group of a serine or threonine residue (Ten Hagen et al., 2003). Triglyceride levels are modulated by GALNT2 as well through its regulatory effects on high-density lipoprotein cholesterol (Roman et al., 2015). Increased *GALNT2* expression in the dietary groups supplemented with VE, ALA, and combination of VE and ALA will positively regulate nutrient transport by influencing lipid metabolism. Interestingly, WB development has been previously hypothesized to be associated with the dysregulation fatty acid metabolism (Abasht et al., 2019; Brothers et al., 2019; Lake and Abasht, 2020). Chapter 5 identified that supplementation of VE and ALA decreased the incidence and severity of WB in broilers at 2 and 3 weeks of age. The higher WB severity along with a higher ileal *GALNT2* expression in the control group in the current study suggests that WB severity may be associated with dysregulated intestinal lipid metabolism.

Both MUC2 and COL4A1 are associated with gut barrier integrity. The intestinal mucus layer is primarily composed of mucin glycoproteins and serves as a barrier protecting the intestinal epithelium from the pathogens (Deplancke and Gaskins, 2001; Velcich et al., 2002). Mucin 2 plays a key role in maintaining the thickness of the intestinal

mucus layer contributing to the barrier structure and nutrient absorption (Uni et al., 2003; Horn et al., 2009). Supplementation of VE and combination of VE and ALA had higher expression of *MUC2* at 3 weeks of age compared to the control suggestive of improved intestinal barrier integrity and function. Another gene involved in gut barrier integrity is *COL4A1*. Type IV collagen is a triple helix composed of two α1 chains and one α2 chain (Qian and Glanvillie, 1984). The repetitive tripeptide sequence of the triple helix is consisted with amino acids Gly-X-Y repeats, in which X and Y can be any amino acids (Trüeb et al., 1982). Type IV collagen is the primary component of the basement membrane which supports the epithelial cells in the gut (Brazel et al., 1987). It forms a network interacting with other extracellular matrix molecules like proteoglycans and non-collagenous glycoproteins (Vercellotti et al.,1985; Basson, 2003). Through these complex interactions, type IV collagen is involved in various cellular activities such as cell adhesion and cell migration (Herbst et al., 1988; Chelberg et al., 1989). Increased *COL4A1* expression in the broilers supplemented with VE, ALA, and combination of VE and ALA at 2 weeks of age is a potential indicator of improved basement membrane integrity. Combination of VE and ALA with the highest expression of *COL4A1* suggested the most improvement on the basement membrane structure. This is consistent with ileal morphology that basement membrane structure was better defined in the dietary treatments especially in the combination of VE and ALA group than the control group. With improved basement membrane structure, the epithelium will be adhered more strongly resulting in increased nutrient absorption and transport function. It was found that VE and ALA

supplementation reduced the WB severity from 1 to 3 weeks of age with combination of VE and ALA having the most significant effect (Chapter 5). This is similar to the current study in which a similar trend was observed with ileal *COL4A1* expression and the basement membrane structure, suggesting that ileal barrier integrity may be closely related with WB development.

In terms of inflammation related attributes, IELs were significantly decreased in ALA and combination of VE and ALA groups at 2 weeks of age and in all of the dietary treatments at 3 weeks of age compared to the control group. This is in agreement with previous study that VE and polyunsaturated fatty acids supplementation decreased IELs in the broilers at 58 day (Chapter 4). The IELs are interspersed in intestinal epithelial cell layer providing various regulatory functions including cytokine production for the mucosal immune system (Yamamoto et al., 1998; Kakar et al., 2003). The intestinal IELs and their released cytokines activate the subsequent protective immune functions (Kakar et al., 2003; Rieger et al., 2015). The IELs are the site of where *LITAF* is expressed (Ateya et al., 2019). As a pro-inflammatory cytokine, LITAF can be up-regulated mediating the host immunity against pathogens (Hong et al., 2006). Same with the IELs, *LITAF* was decreased expressed in VE, ALA, and combination of VE and ALA groups at 3 weeks of age compared to the control group. The decreased IELs and *LITAF* expression in the dietary treatments suggests that VE and ALA both independently and in combination reduced intestinal inflammation at 2 and 3 weeks of age. The combination of VE and ALA showed the most significant

effect on reducing ileal inflammation because of its lowest expression of *LITAF* at 3 weeks of age.

The ileal morphological attributes were consistent with the expression of genes associated with gut nutrient transport, barrier integrity, and inflammation indicating positive effects of VE and ALA supplementation on improving gut health and nutrient absorption, with the combination of VE and ALA showing a better performance than supplementation of VE or ALA independently. This is consistent with plasma α-tocopherol concentration in the current study. Plasma VE concentration in the combination of VE and ALA group was higher than in the VE group at 3 weeks of age, which suggests that plasma VE was more efficiently accumulated in the broilers when ALA was combined supplemented with VE. After absorbed into the gastrointestinal tract in the broilers, ALA is rapidly reduced to dihydrolipoic acid which can react with reactive oxygen species and enhance anti-oxidative enzymes (Bjørklund et al., 2019). In addition, dihydrolipoic acid can recycle VE from its oxidized form by reducing glutathione disulphide, dehydroascorbate, and semidehydroascorbyl radical, and ubiquinone (Sohaib et al., 2018). This results in the synergistic function of VE and ALA on preventing oxidative stress. As oxidative stress can trigger multiple inflammatory pathways, combination of VE and ALA showed synergistic effects on reducing inflammation as well.

Ileal morphology and gene expression were correlated with broiler final body weight, p. major muscle weight, fiber width, and gene expression. Broiler final body weight, p. major muscle weight, morphology score with a higher score representing more

well-structured muscle fiber, and fiber width were positively correlated with ileal morphology and expression of genes associated with nutrient transport. The positive correlations between final body weight, p. major muscle weight, morphology score, fiber width and ileal morphology and gene expression were also found in broilers at market age (Chapter 4). The positive correlations suggest that improved ileal structure, nutrient absorption, and transport function have a beneficial influence on total growth performance and breast muscle development. Expression of *PPARγ* and *SELE* in the p. major muscle were negatively correlated with ileal villus height and the ratio of villus height to crypt depth. The *PPARγ* is an adipogenic gene regulating lipid metabolism (Hu et al., 1995; Kliewer et al., 1997; Rosen et al., 1999). Expression of *PPARγ* is an important maker for the development of WB as WB has been linked with dysregulation of lipid deposition (Abasht et al., 2019; Brothers et al., 2019; Lake and Abasht, 2020). The negative correlations between p. major muscle *PPARγ* expression and the ileal morphological attributes indicate that the dysregulated lipid metabolism of the p. major muscle is related with the impaired ileal morphology. This suggests that the WB development may be closely associated with intestinal development. Selectin E is involved in chronic and acute inflammation (Lundberg, 2000; Ley, 2003). The negative correlations between p. major muscle *SELE* expression and the ileal villus height and the ratio of villus height to crypt depth indicate that reduced inflammatory level in p. major muscle was related with improved ileal morphology. This is suggestive that inflammation in p. major muscle has a negative influence on intestinal morphology associated with nutrient absorption. The close

correlations between p. major muscle morphology and expression of adipogenic and pro-inflammatory genes, and ileal morphology and expression of genes related with nutrient transport strongly suggest that WB development is influenced by gut health.

In conclusion, supplementation of VE and ALA independently and in combination showed a beneficial effect on improving intestinal morphology at 1, 2, and 3 weeks of age. Genes involved in gut nutrient transport, barrier integrity, and inflammation were differentially expressed in VE, ALA, and combination of VE and ALA groups at 2 and 3 weeks of age compared to the control group. Combination of VE and ALA supplementation showed a more beneficial influence on ileal morphology and expression of genes associated with barrier integrity and inflammation than VE and ALA supplementation independently at 1, 2, and 3 weeks of age. Ileal morphology and gene expression were closely correlated with broiler final body weight, p. major muscle weight, morphology and gene expression. Research focused on the mechanism of intestinal development targeting lipid metabolism and responses to inflammation identified in the current study in WB affected and unaffected broilers will be addressed in future studies.

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Table 6.1 Primer sequences for real-time quantitative PCR

Gene	Accession number	Forward primer (5' to 3')	Reverse primer (5' to 3')	Amplicon (bp) size
Nutrient transport				
SLC15A[1]	NM_204365.1	TCCCATGGAG TCAACAGGCT	GCTAGAAACA ATGCCGGCTG	160
GALNT2[2]	XM_015284386.2	GCAGGAAGGA GGACCCAAAC	GATCCTGACC AGATCGCACC	171
Gut barrier integrity				
MUC2[3]	NM_001318434.1	CTGGAAGGTT GCTACCCCAG	CTCAATGGAT CCTGAGGGGC	183
COL4A1[4]	NM_001162399.3	CTAGGGCCTC CAGGTGTT	AAGGCCCTGT TACTCCTTGC	247
Inflammation				
LITAF[5]	NM_204267.1	TGTGGGGCGT GCAGTG	ATGAAGGTGG TGCAGATGGG	194
IFNG[6]	NM_205149.1	ATGTAGCTGA CGGTGGACCT	TCAAGTCGTTC ATCGGGAGC	246
Housekeeping gene				
GAPDH[7]	U94327.1	GAG GGT AGT GAA GGC TGC TG	CCACAACACG GTTGCTGTAT	175

[1]SLC15A1 = Solute carrier family 15 member 1.
[2]GALNT2 = Polypeptide N-acetylgalactosaminyltransferase 2.
[3]MUC2 = Mucin 2.
[4]COL4A1 = Collagen type IV alpha 1 chain.
[5]LITAF = Lipopolysaccharide-induced tumor necrosis factor-alpha factor.
[6]IFNG = Interferon gamma.
[7]GAPDH = Glyceraldehyde-3-phosphate dehydrogenase.

Table 6.2 Effect of vitamin E and alpha lipoic acid on broiler plasma α-tocopherol concentration

Item (μM)	Age (week)	Treatments[1]				SEM[2]	*P*-Value
		Control	VE	AlA	VE and ALA		
Plasma α-tocopherol	1	26.12[b]	92.18[a]	36.28[b]	90.42[a]	7.54	< 0.01
	2	21.24[b]	86.24[a]	31.87[b]	89.29[a]	7.36	< 0.01
	3	20.02[c]	57.70[b]	23.74[c]	69.43[a]	3.02	< 0.01

[a-c]Means within a row without a common letter are significantly different ($P \leqslant 0.05$).
[1]Values are means of 10 pens per treatment, each pen included three birds. Broilers in the control group were fed corn-soybean meal basal diet. Vitamin E (VE; 160mg/kg) and alpha lipoic acid (ALA; 500 mg/kg) were supplemented independently and in combination in the dietary treatments during the entire study (0 to 21 d). At 1, 2 and 3 weeks of age, one chick from each pen was harvested.
[2]SEM = Standard error of means.

Table 6.3 Effect of vitamin E and alpha lipoic acid on broiler ileal morphology

Item	Age (week)	Treatments[1]				SEM[2]	*P*-Value
		Control	VE	AlA	VE and ALA		
Villus height (μm)	1	400.66[d]	419.66[c]	451.23[a]	433.25[b]	3.47	<0.01
	2	533.19[b]	524.61[b]	525.27[b]	547.27[a]	3.15	<0.01
	3	623.88[b]	624.99[b]	648.52[a]	647.2[a]	14.66	<0.01
Crypt depth (μm)	1	76.63	74.75	73.16	80.81	3.51	0.46
	2	89.41	84.33	84.13	86.35	3.59	0.69
	3	92.60	93.07	99.14	87.78	4.95	0.38
Villus/crypt[3]	1	5.33	5.71	6.27	5.47	0.29	0.14
	2	6.03	6.27	6.36	6.39	0.25	0.74
	3	6.76	6.82	6.57	7.51	0.28	0.13
Villus width (mm^2)	1	128.19	112.49	114.25	128.94	6.28	0.14
	2	121.06	124.18	125.77	114.79	4.86	0.4
	3	132.42	139.09	130.99	125.13	8.33	0.74
Surface area (mm^2)	1	0.16	0.15	0.16	0.18	0.01	0.55
	2	0.20	0.21	0.21	0.19	0.01	0.96
	3	0.26	0.27	0.26	0.24	0.02	0.48
Distance between villi (μm)	1	11.43	11.17	10.25	10.81	0.88	0.8
	2	13.84	13.07	12.83	11.31	0.79	0.20
	3	13.19	14.47	14.35	14.2	0.88	0.75
IELs[4]	1	11.99	12.17	12.67	11.67	2.11	0.78
	2	16.11[a]	16.00[a]	13.52[b]	14.17[b]	0.45	<0.01
	3	13.61[a]	12.33[b]	11.67[b]	11.50[b]	0.41	<0.01
Goblet cell[5]	1	10.07	10.39	10.95	10.92	0.48	0.51
	2	9.26	9.95	9.47	9.63	0.42	0.73
	3	9.89	10.25	10.35	10.54	0.47	0.79

[a-d]Means within a row without a common letter are significantly different ($P \leqslant 0.05$).
[1]Values are means of 10 pens per treatment, each pen included three birds. Broilers in the control group were fed corn-soybean meal basal diet. Vitamin E (VE; 160mg/kg) and alpha lipoic acid (ALA; 500 mg/kg) were supplemented independently and in combination in the dietary treatments during the entire study (0 to 21 day). At 1, 2 and 3 weeks of age, one chick from each pen was harvested.
[2]SEM = Standard error of means; [3]Villus/crypt = Ratio of villus height to crypt depth.
[4]IELs = Number of intraepithelial lymphocyte per 100 epithelial cells.
[5]Goblet cell = Number of goblet cell per 100 μm of the villi.

Table 6.4 Effect of vitamin E and alpha lipoic acid on broiler ileal gene expression

Item	Age (week)	Treatments[1]				SEM[2]	*P*-value
		Control	VE	ALA	VE and ALA		
Nutrient transport							
SLC15A1[3]	1	54.90	53.74	55.41	55.52	1.05	0.58
	2	71.41^{c}	73.49^{bc}	76.33^{ab}	77.22^{a}	1.34	0.01
	3	70.85^{b}	74.43^{a}	74.77^{a}	75.10^{a}	1.19	0.03
GALNT2[4]	1	2.72	2.81	2.75	2.66	0.10	0.42
	2	3.19^{b}	3.88^{a}	3.45^{ab}	3.76^{a}	0.16	0.02
	3	4.05^{b}	4.30^{ab}	4.59^{a}	4.49^{a}	0.15	0.02
Gut barrier integrity							
MUC2[5]	1	30.46	33.92	30.73	34.43	1.69	0.22
	2	62.07	65.43	64.50	63.35	2.21	0.72
	3	141.79^{c}	164.75^{a}	148.37^{bc}	155.21^{ab}	4.31	0.01
COL4A1[6]	1	77.31	78.76	80.19	80.23	1.23	0.28
	2	195.79^{c}	209.12^{b}	207.09^{b}	218.30^{a}	3.87	<0.01
	3	132.22^{b}	133.56^{b}	134.97^{ab}	138.24^{a}	2.51	0.04
Inflammation							
LITAF[7]	1	1.18	1.19	1.12	1.16	0.05	0.81
	2	8.16	8.15	8.07	8.14	0.44	0.86
	3	17.74^{a}	15.39^{b}	12.85^{c}	12.03^{d}	0.81	<0.01
IFNG[8]	1	1.20	1.18	1.18	1.14	0.04	0.59
	2	4.26	4.30	4.13	4.18	0.18	0.82
	3	4.11	4.10	4.09	3.97	0.13	0.84

[a-c]Means within a row without a common letter are significantly different ($P \leq 0.05$).
[1]Values are means of 10 pens per treatment, each pen included three birds. Broilers in the control group were fed corn-soybean meal basal diet. Vitamin E (VE; 160mg/kg) and alpha lipoic acid (ALA; 500 mg/kg) were supplemented independently and in combination in the dietary treatments during the entire study (0 to 21 day). At 1, 2 and 3 weeks of age, one chick from each pen was harvested.
[2]*SEM* = Standard error of means.
[3]*SLC15A1* = Solute carrier family 15 member 1.
[4]*GALNT2* = Polypeptide N-acetylgalactosaminyltransferase 2.
[5]*MUC2* = Mucin 2; [6]*COL4A1* = Collagen type IV alpha 1 chain.
[7]*LITAF* = Lipopolysaccharide-induced tumor necrosis factor-alpha factor.
[8]*IFNG* = Interferon gamma.

Table 6.5 Correlation coefficients for ileal and breast muscle morphology and gene expression[1]

	Villus height	Crypt depth	Villus/ crypt[2]	Surface area	*MUC2*[3]	*SLC15A1*[4]	*GALNT2*[5]
Final body weight							
Pearson	0.96	0.53	0.44	0.67	0.53	0.23	0.21
P-value[6]	<0.01	<0.01	<0.01	<0.01	<0.01	0.02	0.02
P. major muscle weight							
Pearson	0.93	0.51	0.45	0.64	0.51	0.20	0.18
P-value	<0.01	<0.01	<0.01	<0.01	<0.01	0.04	0.05
Morphology score[7]							
Pearson	0.40	0.19	0.15	0.20	0.25	0.04	0.19
P-value	<0.01	0.05	0.11	0.03	0.01	0.69	0.04
Fiber width							
Pearson	0.84	0.43	0.40	0.56	0.39	0.15	0.16
P-value	<0.01	<0.01	<0.01	<0.01	<0.01	0.14	0.09
PPARγ[8]							
Pearson	-0.38	-0.12	-0.30	-0.15	-0.18	-0.05	-0.01
P-value	<0.01	0.28	0.01	0.18	0.10	0.66	0.97
SELE[9]							
Pearson	-0.25	0.03	-0.21	0.01	-0.10	0.01	0.12
P-value	0.02	0.79	0.05	0.91	0.35	0.98	0.24

[1]Perason correlation coefficient for ileal morphology and gene expression (in columns) and pectoralis major muscle (p. major muscle; breast muscle) morphology and differentially expression genes (in rows).
[2]Villus/crypt=Ratio of villus height to crypt depth.
[3]*MUC2* = Mucin 2.
[4]*SLC15A1* = Solute carrier family 15 member 1.
[5]*GALNT2* = Polypeptide N-acetylgalactosaminyltransferase 2.
[6]*P*-value for each Pearson correlation coefficient.
[7]Morphology scoring scale of one to five was used for p. major muscle overall morphology evaluation. Samples with limited or no perimysial or endomysial connective tissue space, and excessive myofiber degradation were given a score of one. Samples with morphology score of five have ample perimysial and endomysial connective tissue spacing, and well-structured muscle fibers. Score of two to four are intermediate.
[8]*PPARγ* = Peroxisome proliferator-activated receptor gamma.
[9]*SELE* = Selectin E.

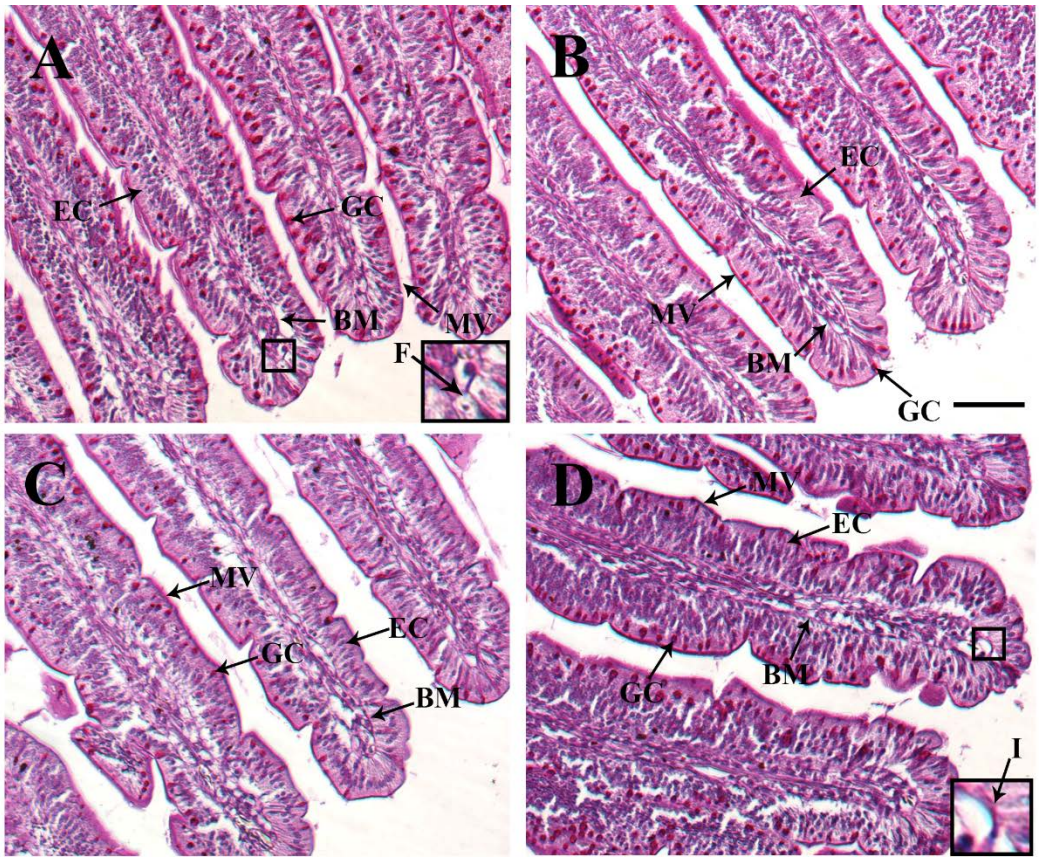

Figure 6.1 Representative photomicrographs of the broiler ileum at 2 weeks of age in the four treatments. Broilers in the control group (A) were fed corn-soybean meal basal diet. Vitamin E (VE; 160mg/kg) was supplemented in the VE group (B) and alpha lipoic acid (ALA; 500 mg/kg) was supplemented in the ALA group (C). Combination of VE and ALA was supplemented in the VE and ALA group (D). BM = Basement membrane; EC = Epithelial cell; F = Fissures in BM; GC = Goblet cell; I = Integrate BM; MV = Microvilli. The boxes contain enlargements of the basement membrane. Scale bar = 50 μm.

Chapter 7: Discussion and Conclusion

7.1 Introduction

With broilers being continuously selected for higher growth rate and breast meat yield, meat myopathies have arisen in breast muscle of commercial fast growing broilers (Dransfield and Sosnicki, 1999; Petracci et al., 2013; Tijare et al., 2016). Among the myopathies, Wooden Breast (WB) has been discovered worldwide (Sihvo et al., 2014; Tasoniero et al., 2016; Xing et al., 2019) resulting in serious economic loss (Kuttappan et al., 2016). Wooden Breast affected muscles are palpably hard (Sihvo et al., 2014) under severe oxidative stress (Mutryn et al., 2015; Abasht et al., 2019; Brothers et al., 2019) and inflammation (Mutryn et al., 2015; Zambonelli et al., 2017). To reduce the incidence and severity of WB and similar myopathies, some are pushing to transition to slower growing broilers (Gocsik et al., 2016; Saatkamp et al., 2019). Slower growing birds take at least two additional weeks to attain the market weight compared to the conventional birds (Fanatico et al., 2005). Although longer production time may alleviate myopathies to some degree, slower growing birds require additional resources such as land, feed, and water, which costs producers more than the conventional birds (Conner, 2010). Such production practices are also not sustainable given the need for increased resources and negative impact on the environment (van de Kerkhof et al., 2010). Alternatively, if we can identify the nutritional strategies that can reduce WB in fast-growing broilers, this would allow us to continue using fast growing broilers without the negative side-effects that are associated with myopathies like WB. In addition, posthatch muscle growth is dependent on satellite

cells, which are most active the first week posthatch (Halevy et al., 2000) and are sensitive to nutritional changes (Halevy et al., 2000; Velleman et al., 2010; Powell et al., 2014). Nutritional strategies can be applied during the early posthatch period having beneficial effects on muscle growth (Noy and Sklan, 1999; Dangott et al., 2000). The overall hypothesis was that WB incidence and severity is influenced by nutritional strategies during the early posthatch period. The overall objective of this study was to reduce the incidence and severity of WB myopathy through early posthatch nutritional interventions including vitamin E (VE) and alpha lipoic acid (ALA) with antioxidant properties, and omega-3 (n-3) fatty acids with anti-inflammatory effects.

7.2 Effect of Vitamin E on Reducing Wooden Breast

Vitamin E has powerful antioxidant capacities (Rymer and Givens, 2010; Cheng et al., 2016; El-Senousey et al., 2018) that can terminate lipid peroxidation process by reacting with free radicals and mitigate cell membrane oxidation (Niki et al., 1993). Since WB affected broilers are under severe oxidative stress (Mutryn et al., 2015; Abasht et al., 2019; Brothers et al., 2019), VE was used in the current study to identify its effects on reducing the incidence and severity of WB.

In general, VE supplementation reduced WB severity without negatively impacting growth performance and meat yield both at an early age (21 days of age; Chapter 5) and at market age (58 days of age; Chapter 2). No influence on growth performance and meat yield was found which is in agreement with previous studies where neither body weight

nor meat yield were affected by dietary VE (Bartov and Frigg, 1992; Sakamoto et al., 2006; Kuttappan et al., 2012; Cheng et al., 2018). This could be due to the fact that the contents of VE in corn-soybean meal basal diets were sufficient to meet the requirement for growth performance and meat production and would not be changed by VE supplementation. In contrast, WB severity evaluated by palpation of p. major muscle was reduced when VE was supplemented during the starter (0 to 10 days) or grower (11 to 24 day) phase in broilers at market age (Chapter 2). The effect of VE on reducing WB severity could be due to its antioxidant potential. Vitamin E can incorporate into the cell membrane where oxidation is initiated (Bou et al., 2009) preventing cells and tissues from oxidative damage (Voljč et al., 2011). Microscopically, VE supplementation during the grower phase had a 16.19% increase of muscle with no WB compared to the control group in broilers at market age (Chapter 3). This beneficial effect of VE on reducing WB severity was initiated as early as 2 weeks of age as supplementation of VE reduced microscopic changes associated with WB at 2 and 3 weeks of age compared to the control group (Chapter 5).

The reduced incidence and severity of WB through VE supplementation is closely associated with muscle and intestinal growth and development, inflammatory state, and lipid metabolism in the p. major muscle and intestine. With regard to muscle growth and development, *myogenic determination factor 1 (MyoD)* was differentially expressed in the VE group at 3 weeks of age (Chapter 5). Muscle growth after hatch is dependent on satellite cells (Moss and Leblond, 1971), which are most active the first week posthatch and then gradually become quiescent (Halevy et al., 2000). However, satellite cells can be

reactivated to undergo the regeneration process when the muscle is damaged (Schultz, 1989). They proliferate to increase myofiber number and differentiate by fusing with existing myofibers (Moss and Leblond, 1971). The MyoD and myogenic factor 5 transcriptional regulatory factors are necessary for proliferation (Rudnicki et al., 1993) while myogenin (MyoG) is required for differentiation leading to the formation of multinucleated myotubes (Hasty et al., 1993). The reduction of *MyoD* expression in the VE group compared to the control group at 3 weeks of age are suggestive of decreased proliferation, implying that fewer muscle fibers were damaged and entered the regeneration process. The lower *MyoD* expression along with the reduced microscopic changes associated with WB in the VE group at 3 weeks of age are in agreement with Velleman and Clark (2015) that expression of genes related with myogenic proliferation are increased in the WB affected breast muscle.

In terms of intestinal growth and development, villus height as an important morphological attribute for nutrient absorption and transport was increased in the VE group beginning at 1 week of age compared to the control (Chapter 6). The digestive and absorptive capacities are enhanced with an increased villus height through higher surface area of nutrient absorption and brush border enzyme expression (Yamauchi et al., 1996). The increased villus height in VE group is suggestive of an improvement in ileal morphology, nutrient uptake and absorptive efficiency beginning at 1 week of age through the VE supplementation. With improved intestinal structure, nutrients can be more efficiently absorbed in small intestine, transported through circulatory system to the liver,

processed in the liver, and incorporated into muscle cells (Hocquette et al., 1998), which could result in better muscle growth and potentially reduce the severity of WB. In the current study, the enhanced intestinal morphology is parallel with improved muscle growth implying that dietary VE reduced WB severity in broilers at market age through improving both p. major muscle and small intestinal growth and development at an early age.

As for inflammation related attributes, VE supplementation decreased intestinal intraepithelial lymphocytes (IELs) at 3 weeks of age compared to the control group (Chapter 6). The IELs provide various regulatory functions including cytokine production for the mucosal immune system (Yamamoto et al., 1998; Kakar et al., 2003) that activate the subsequent protective immune functions (Kakar et al., 2003; Rieger et al., 2015). Intestinal expression of *lipopolysaccharide-induced tumor necrosis factor-alpha factor (LITAF)* associated with inflammation was decreased in the VE group at 3 weeks of age compared to the control (Chapter 6). As a pro-inflammatory cytokine, LITAF can be up-regulated mediating the host immunity against pathogens (Hong et al., 2006). The decreased intestinal IELs number and *LITAF* expression in the VE group suggest that VE reduced intestinal inflammation at 2 and 3 weeks of age. The reduced intestinal inflammation likely contributed to the decreased WB severity as WB affected tissues are under severe inflammation (Mutryn et al., 2015; Zambonelli et al., 2017).

Additionally, expression of genes involved in lipid metabolism such as *peroxisome proliferator-activated receptor gamma (PPARγ)* and *CCAAT/enhancer binding protein alpha (CEBPα)* were reduced in the p. major muscle at 2 and 3 week of age, suggesting

reduced fat deposition in the p. major muscle (Hu et al., 1995; Kliewer et al., 1997; Rosen et al., 1999). Similar findings in lipid metabolism were found in the small intestine in the current study. Intestinal expression of *polypeptide N-acetylgalactosaminyltransferase 2 (GALNT2)* associated with nutrient transport was increased in VE group at 3 weeks of age compared to the control. Triglyceride levels can be modulated by GALNT2 through its regulatory effects on high-density lipoprotein cholesterol (Roman et al., 2015). Increased *GALNT2* expression in the ALA group can positively regulate nutrient transport by influencing lipid metabolism. These data suggest the reduced fat deposition in the p. major muscle could be related with the improved intestinal nutrient transport and less dysregulated lipid metabolism in small intestine, which contribute to the decreased severity of WB.

Thus, VE supplementation during the starter phase or grower phase reduced WB severity in broilers at market age. This beneficial effect on reducing WB was initiated as early as 2 weeks of age. Improved muscle and intestinal growth and development, reduced inflammation and dysregulated lipid metabolism through VE supplementation in p. major muscle and small intestine in broilers at an early age likely results in improved muscle and intestinal structure and a lower incidence and severity of WB in broilers at market age.

7.3 Effect of Omega-3 Fatty Acids on Reducing Wooden Breast

Omega-3 fatty acids are polyunsaturated fatty acids (PUFA) with anti-inflammatory effects (Simopoulos, 2002; Calder, 2003; Rahimi et al., 2011; Yu et al.,

2018). Chapter 2 to 4 evaluated the effect of n-3 fatty acids early posthatch on reducing the WB severity. However, n-3 fatty acids did not show beneficial effect on reducing WB in broilers at market age (Chapter 2). In contrast, n-3 fatty acids supplementation in starter diets significantly decreased final body weight, hot carcass weight, and chilled carcass weight. This is consistent with previous studies (Ayerza et al., 2002; Azcona et al., 2008; Navidshad, 2009). Hulan et al. (1988) attributed the reduced growth performance to the reduced palatability and higher calcium levels of fish oil supplementation. However, several studies using fish oil in the diets did not find adverse effects on body weight (Lo´pez-Ferrer et al., 2001; Farhoomand and Checaniazer, 2009). The contrasting results could be related to the concentration of PUFA with higher PUFA levels more likely having negative impact on growth performance and meat yield. As for meat quality, Wood et al. (2003) reported that fatty acid composition influences meat quality attributes such as hardness. They suggest that the effect on hardness is due to the different melting points of the fatty acids in meat with higher levels of unsaturated fatty acids having lower melting points and softer meat. This is in agreement with the current study that shear force in n-3 fatty acids supplementation group with higher PUFA levels during the grower phase was lower than VE supplementation during the grower phase.

When n-3 fatty acids were supplemented independently, it did not show effects on reducing WB as beneficial as VE did (Chapter 2 and 3). The percentages of p. major muscles with no WB and mild WB were higher in VE supplementation group during the grower phase compared to the n-3 fatty acids supplementation group during the grower

phase (Chapter 3). This could be related with lipid and glycolytic metabolism in the p. major muscle (Chapter 3) and the intestinal basement membrane integrity (Chapter 4). Dysregulated lipid metabolism has been closely associated with WB (Abasht et al., 2019; Brothers et al., 2019; Lake et al., 2019). Higher fat content along with a higher severity of WB in the p. major muscle was identified in n-3 fatty acids supplementation group during the starter phase compared with VE supplementation during the starter phase. Genes related with lipid and glycolytic metabolism were differentially expressed between VE and n-3 fatty acids groups as well. Lactate dehydrogenase A (LDHA) is an enzyme regulating conversion between lactate and pyruvate (Cahn et al., 1962). Thep. major muscle is composed of type 2B fibers, which is an anaerobic muscle fiber, lactic acid is produced by glycolytic metabolism. When available space for circulatory system is restricted due to selection for higher muscle mass and excessive muscle fiber hypertrophy (Dransfield and Sosnicki, 1999), lactic acid cannot be removed sufficiently resulting in decreased pH and muscle damage (Velleman et al., 2003). Expression of *LDHA* in the p. major muscle was increased in the broilers supplemented with VE in the grower diet compared to n-3 fatty acids supplementation in the starter diet. Increased levels of *LDHA* indicates a higher potential of lactate and pyruvate conversion and thus reducing the muscle damage and resulting in less severity of WB through VE supplementation compared to n-3 fatty acids supplementation. This is parallel with Zhao et al. (2020) that *LDHA* expression level was decreased in WB affected tissues. In addition, intestinal expression of *collagen type IV alpha 1 chain (COL4A1)* associated with ileal basement membrane integrity was increased

in the broilers supplemented with VE in the starter diet compared to n-3 fatty acids supplementation during the grower phase as shown in Chapter 4. Collagen type IV is a critical component of gut basement membrane (Zhang et al., 2003). Higher expression of *COL4A1* in the group supplemented with VE during the starter phase indicates improved integrity of basement membrane. With improved gut structure, broilers have higher nutrient uptake and absorptive efficiency, which could lead to more nutrients incorporating into muscle improving muscle structure and meat quality (Hocquette et al., 1998).

Interestingly, the negative effects of n-3 fatty acids on growth performance and meat yield were not shown when VE was combined supplemented with n-3 fatty acids (Chapter 2). Similar results were found in the p. major muscle and small intestine as shown in Chapter 3 and 4, respectively. Expression of *syndecan-4* in the p. major muscle was increased in broilers fed a combination of VE and n-3 fatty acids in the grower diet compared to n-3 fatty acids supplementation in the grower diet and the control group. Syndecan-4 is a transmembrane heparan sulfate proteoglycan involved in focal adhesion formation and cell migration (Longley et al., 1999; Couchman, 2003; Song et al., 2012b; Shin et al., 2013). It regulates focal adhesions by activating protein kinase C alpha through the syndecan-4 cytoplasmic domain (Woods and Couchman, 1992; Lee et al., 1998; Lim et al., 2003; Song et al., 2012a; Shin et al., 2013). Increased expression of *syndecan-4* in the p. major muscle in the group supplemented with VE and n-3 fatty acids in the grower diet is suggestive of improved cell migration, which is necessary for the muscle repair and regeneration process. In the ileum, IELs number was not different between n-3 fatty acids

supplementation group during the grower phase compared to the control while was lower in combination of VE and n-3 fatty acids group during the grower phase compared to the control. This suggests that combination of VE and n-3 fatty acids had a higher ability of reducing inflammation than n-3 fatty acids supplementation independently. As VE can reduce lipid peroxidation (Konieczka et al., 2018), VE and n-3 fatty acids work synergistically reducing the negative effect of n-3 fatty acids on broiler physiology both in the p. major muscle and in the intestine.

Overall, n-3 fatty acids did not show effect on reducing WB severity while reduced growth performance and meat yield. When VE was combined added with n-3 fatty acids in broiler diets, the negative effects were reduced but still did not show beneficial effects on WB severity compared to the control. Therefore, n-3 fatty acids were not further studied in the current study. Instead, another fatty acid with an anti-inflammatory effect, ALA, was used to determine its effect on reducing WB.

7.4 Effect of Alpha Lipoic Acid on Reducing Wooden Breast

Alpha lipoic acid is short chain fatty acid with both antioxidant and anti-inflammatory properties (Li et al., 2014; Ma et al., 2015). Chapter 5 and 6 evaluated the effects of ALA on the developmental onset, severity, and progression of the WB myopathy during the early posthatch period based on developmental changes in p. major muscle and intestinal morphological structure and gene expression in broilers.

In terms of growth performance, dietary ALA did not have an impact on body weight and p. major muscle weight. However, supplementation of ALA decreased feed intake at 3 weeks of age. This is in agreement with a previous study indicating that ALA supplementation reduced growth performance (Zhang et al., 2014). Zhang et al. (2014) attributed the impaired growth performance to poor palatability due to the disulfide bond in ALA, which suppresses feed intake. It has been reported that 800 and 1200 mg/kg ALA added to the diets decreased broiler feed intake but 400 mg/kg ALA did not have an influence on feed intake and feed conversion ratio (El-Senousey et al., 2013). The differences in feed intake could be because different concentrations of ALA have varied effects on feed intake with higher levels of ALA having a higher potential negative effect.

Alpha lipoic acid supplementation decreased microscopic changes associated with WB at 2 and 3 weeks of age compared to the control group as shown in Chapter 5. The reduced WB severity could be through influencing muscle growth and development, regulating inflammation and lipid metabolism in both p. major muscle and small intestine, and improving intestinal nutrient absorption and transportation functions.

With regard to muscle growth and development, genes associated with muscle proliferation and differentiation, *MyoD and MyoG*, were differentially expressed in the ALA group at 3 weeks of age. During muscle regeneration process activated by muscle fiber damage, MyoD and myogenic factor 5 transcriptional regulatory factors are necessary for muscle cell proliferation (Rudnicki et al., 1993) while MyoG is required for differentiation leading to the formation of multinucleated myotubes (Hasty et al., 1993).

The reduction in *MyoD* and *MyoG* expression in ALA group at 3 weeks of age are suggestive of decreased proliferation and differentiation, suggesting that fewer muscle fibers were damaged and entered the muscle repair process. The higher *MyoD* and *MyoG* expression along with the higher microscopic WB severity in the control group compared to the ALA group at 3 weeks of age are in agreement with Velleman and Clark (2015) that expression of genes related with myogenic proliferation and differentiation was increased in the WB affected breast muscle.

Inflammation related attributes were changed due to ALA supplementation. In the p. major muscle, expression of *selectin E (SELE*), associated with oxidative stress and inflammation, was decreased at 2 and 3 weeks of age when the diets were supplemented with ALA compared to the control group. As an important adhesion molecule, SELE plays a key role in chronic and acute inflammation (Lundberg, 2000; Ley, 2003) and helps mediate leukocyte migration to inflammatory sites through leukocyte-endothelial interactions (Fries et al., 1993; de Lucass et al., 1994). Reduced inflammation was also observed in in the small intestine in the current study. The ileal IELs number were significantly decreased in the ALA group at 2 and 3 weeks of age compared to the control group. The IELs are the site of where lipopolysaccharide-induced tumor necrosis factor-alpha factor (LITAF) is expressed (Ateya et al., 2019). As a pro-inflammatory cytokine, LITAF can be up-regulated mediating the host immunity against pathogens (Hong et al., 2006). As with the IELs, ileal *LITAF* expression was decreased in ALA group at 3 weeks of age compared to the control. These data suggest that inflammation in the p. major muscle

could be associated with inflammatory state in the small intestine, contributing to the onset of WB.

Alpha lipoic acid not only reduced inflammation but also reduced dysregulated lipid deposition in the p. major muscle. Dysregulation of lipid deposition has been found to be closely related with WB (Abasht et al., 2016; Papah et al., 2017; Zambonelli et al., 2017; Lake et al., 2019). Both *PPARγ* and *CEBPα* mediating differentiation of muscle cells into adipocytes (Hu et al., 1995) and fatty acids metabolism (Kliewer et al., 1997; Rosen et al., 1999) were decreased in ALA group at 2 and 3 weeks of age. Higher expression of *PPARγ* and *CEBPα* is commonly related with increased intramuscular lipid deposition (Liu et al., 2017; Cui et al., 2018). In the present study, broilers supplemented with ALA had decreased *PPARγ* and *CEBPα* expression, suggesting reduced fat deposition associated with WB in the p. major muscle.

The reduced WB severity through ALA supplementation could also be attributed to improved nutrient absorption and transportation in the gut. Villus height was increased in the ALA group beginning at 1 week of age compared to the control group. The digestive and absorptive capacity can be enhanced with increased villus height (Yamauchi et al., 1996). The increased villus height in the ALA group is suggestive of an improvement in ileal morphology, nutrient uptake and absorptive efficiency beginning at 1 week of age due to ALA supplementation. In addition, genes associated with gut nutrient transport were differentially expressed in the ALA group. The *solute carrier family 15 member 1 (SLC15A1)* expression was increased in the ALA group at 2 and 3 weeks of age compared

to the control group. The SLC15A1 is also called peptide transporter 1. It belongs to the superfamily proton oligopeptide transporters (Ingersoll et al., 2012) and is responsible for transportation of dipeptides and tripeptides in the enterocytes (Osmanyan et al., 2018). Higher *SLC15A1* expression is suggestive of greater protein digestion and absorption in the broilers supplemented with ALA compared to the control.

Gut barrier integrity was improved by ALA supplementation resulting in improved nutrient absorption and transportation functions. Type IV collagen is the primary component of the basement membrane supporting the epithelial cells in the gut (Brazel et al., 1987). Broilers supplemented with ALA had increased *COL4A1* expression at 2 weeks of age, which suggests improved basement membrane integrity. With improved basement membrane structure, the epithelium can be adhered more strongly resulting in increased nutrient absorption and transport function.

Vitamin E was shown in the current study to work synergistically with ALA on reducing WB severity. Combining VE and ALA had the most significant effect on reducing WB severity compared to VE and ALA supplementation independently at 2 and 3 weeks of age. Moreover, combination of VE and ALA ameliorated the negative influence of ALA on growth performance because of their synergistic effect on alleviating oxidative stress (Srilatha et al., 2010; Parveen et al., 2013).

The synergistic effects of VE and ALA can also be found in small intestine. Combination of VE and ALA group had a higher villus height than VE and ALA supplementation independently at 2 and 3 weeks of age. This is consistent with Yoo et al.

(2016) that combination of VE and ALA increased villus height in broilers with the combination of VE and ALA had a maximal beneficial effect. A higher intestinal expression of *COL4A1* was found in combination of VE and ALA compared to ALA supplementation alone implying the most improvement on the basement membrane structure through VE and ALA combined supplementation. This is consistent with ileal morphology that basement membrane structure was well defined in the dietary treatments especially in the combination of VE and ALA group. Additionally, the combination of VE and ALA showed the most significant effect on reducing ileal inflammation due to its lowest ileal expression of *LITAF* at 3 weeks of age. These all suggest that VE and ALA can be combined supplemented leading to a better effect on reducing the incidence and severity of WB.

The synergistic effect of VE and ALA is supported by the serum VE concentration in the current study (Chapter 6). Serum VE concentration in the combination of VE and ALA group was higher than in the VE group at 3 weeks of age, which suggests that serum VE was more efficiently accumulated in the broilers when ALA was combined supplemented with VE. The higher accumulation could be because VE is a lipid soluble vitamin that reduced the altered lipid metabolism in the gut when ALA is combined resulting in more efficient absorption and metabolism in the intestine. Additionally, ALA can recycle VE from its oxidized form by reducing glutathione disulphide, dehydroascorbate, and semidehydroascorbyl radical, and ubiquinone (Sohaib et al., 2018), resulting in the synergistic function of VE and ALA on preventing oxidative stress.

The findings in the current study showed that dietary VE and ALA especially when they were combined reduced the incidence of WB as early as 2 weeks of age, which can likely be used as a tool to reduce WB incidence and severity in broilers grown to market age.

7.5 Correlation Between Breast Muscle and Small Intestine

The current study showed that there were close correlations between p. major muscle and small intestinal morphology and expression of genes associated with WB. In broilers both at an early age (21 days of age; Chapter 4) and at market age (58 days of age; Chapter 6), body weight, p. major muscle weight, muscle fiber width, and muscle morphology score with a higher score representing more well-structured muscle fibers were positively correlated with ileal morphology and expression of genes associated with nutrient absorption and transport. The positive correlations suggest that improved ileal structure, intestinal nutrient absorption and transport functions have a beneficial influence on broiler growth performance and p. major muscle growth and development.

In broilers at an early age, expression of *PPARγ* and *SELE* in the p. major muscle were negatively correlated with ileal villus height and the ratio of villus height to crypt depth. The *PPARγ* is an adipogenic gene regulating lipid metabolism (Hu et al., 1995; Kliewer et al., 1997; Rosen et al., 1999). Expression of *PPARγ* is an important marker for the development of WB as WB has been linked with dysregulation of lipid deposition (Abasht et al., 2019; Brothers et al., 2019; Lake and Abasht, 2020). The negative

correlations between *PPARγ* expression in the p. major muscle and the ileal morphological attributes suggest that the dysregulated lipid metabolism in p. major muscle is related with the impaired ileal morphology, which implies that the WB development may be closely associated with intestinal development. The negative correlations between expression of *SELE* associated with inflammation in the p. major muscle (Lundberg, 2000; Ley, 2003) and the ileal villus height and the ratio of villus height to crypt depth indicate that reduced inflammatory level in p. major muscle was correlated with improved ileal morphology. This implies that inflammation in p. major muscle has a negative influence on intestinal morphology associated with nutrient absorption. The close correlations between p. major muscle morphology and expression of adipogenic and pro-inflammatory genes, and ileal morphology and expression of genes related with nutrient transport strongly indicates that WB development is influenced by gut health. Thus, early posthatch nutritional interventions to reduce intestinal inflammation and oxidative stress and improve intestinal nutrient absorption and transport would influence breast muscle development as well as WB development.

7.6 Conclusions and Future Direction

Overall, these results indicate that VE supplementation (200 IU/kg) during the starter phase (0 to 10 day) or grower phase (11 to 24 day) reduced the incidence and severity of WB both by palpation and by microscopic evaluation in broilers at market age (58 days of age). Vitamin E supplementation during the grower phase showed a more

beneficial effect on improving muscle and intestinal morphology. Addition of n-3 fatty acids (n-6/n-3 ratio of 30.2:1), however, decreased growth performance and meat yield and did not show positive effects on reducing WB. The dietary effects on reducing the WB severity was initiated from early age. Vitamin E (160 mg/kg) and ALA (500 mg/kg) supplemented independently and in combination had positive effects on mitigating WB severity, improving muscle and intestinal structures as early as 2 weeks of age, with combination of VE and ALA showing the most effective effect.

The current study represents an initial study evaluating the effect of VE, n-3 fatty acids, and ALA on reducing the incidence and severity of WB. Supplementation of VE or combination of VE and ALA have been found to reduce the incidence and severity of WB most effectively compared to the other dietary treatments. At least $32 million of economic loss could be reduced per year through VE and ALA supplementation based on reduced WB incidence and the WB estimated economic loss each year (Kuttappan et al., 2016). However, some studies need to be conducted before the dietary treatments are transferred to the poultry industry. For example, future study should determine the effects of ALA on the developmental onset, severity, and progression of WB of the broilers to an older age. In addition, the most beneficial VE and ALA supplementation concentration and administration period to reduce the severity of WB through altering biological activities of satellite cells to form high quality muscle and improving gut health to reduce intestinal inflammation and improve nutrient absorption in the broilers at market age need to be further investigated.

Other dietary treatments especially the antioxidants will need to be determined too. For example, vitamin C, also known as ascorbic acid, improves antioxidant status and immune function in broilers (Abdel-Fattah, 2007; Leskovec et al., 2018; Min et al., 2018). Ethoxyquin is a quinolone-based antioxidant, which has been shown to improve meat stability (Bartov and Bornstein, 1977) and exert an improving effect on VE status in broilers (Lauridsen et al., 1995). These antioxidants have not been reported to reduce the incidence and severity of WB in broilers and should be evaluated for their potential uses. Additionally, combination of various antioxidants to maximize antioxidant properties should also be considered in the future studies.

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